Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes

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We have studied the C-terminal signal which directs the complete export of the 1024-amino-acid hemolysin protein (HlyA) of *Escherichia coli* across both bacterial membranes into the surrounding medium. Isolation and sequencing of homologous *hlyA* genes from the related bacteria *Proteus vulgaris* and *Morganella morganii* revealed high primary sequence divergence in the three HlyA C-termini and highlighted within the extreme terminal 53 amino acids the conservation of three contiguous sequences, a potential 18-amino-acid amphiphilic α -helix, a cluster of charged residues, and a weakly hydrophobic terminal sequence rich in hydroxylated residues. Fusion of the C-terminal 53 amino acid sequence to non-exported truncated HlyA directed wild-type export but export was radically reduced following independent disruption or progressive truncation of the three C-terminal features by in-frame deletion and the introduction of translation stop codons within the 3' *hlyA* sequence. The data indicate that the HlyA C-terminal export signal comprises multiple components and suggest possible analogies with the mitochondrial import signal. Hemolysis assays and immunoblotting confirmed the intracellular accumulation of non-exported HlyA proteins and supported the view that export proceeds without a periplasmic intermediate. Comparison of cytoplasmic and extracellular forms of an independently exported extreme C-terminal 194 residue peptide showed that the signal was not removed during export.

Key words: amphiphilic helix/hemolysin/protein export/secretion signal

Introduction

Bacterial export of proteins is characteristically initiated by a leader signal sequence which is located at the protein's extreme N-terminus and removed by a cellular peptidase shortly after the precursor proteins translocate across the cytoplasmic membrane (Michaelis and Beckwith, 1982; Wickner and Lodish, 1985). The secretion signal is closely analogous to eukaryotic secretion sequences, comprising on average ~23 residues which determine a short positively charged terminal region, a nonpolar central domain encompassing hydrophobic core residues, and a cleavage site recognized by the signal peptidase. Bacterial secretion based on N-terminal signals is normally dependent upon the

products of several *sec* genes, e.g. *secA*, and does not proceed across the outer membrane, a prohibitive barrier to proteins reaching the bacterial periplasm (Pugsley and Schwartz, 1985).

Export of the 110-kD hemolysin protein (HlyA) by enterobacteria is a remarkable exception to this principle, occurring without an N-terminal signal and directing translocation across not only the cytoplasmic membrane but also the bacterial outer membrane. It is believed to proceed without a periplasmic intermediate and leads to the release of the hemolysin protein into the surrounding medium (Haertlein *et al.*, 1983; Felmlee *et al.*, 1985; Gray *et al.*, 1986; Mackman *et al.*, 1986). The hemolysin export process is independent of *secA* (Mackman *et al.*, 1987) but dependent upon two specific membrane-located secretion proteins, HlyB [a putative ATP-binding analogue of the mammalian P-glycoprotein (Higgins *et al.*, 1986; Gerlach *et al.*, 1986)] and HlyD, which are encoded by genes contiguous and co-expressed with the structural gene *hlyA* (Wagner *et al.*, 1983; Hacker and Hughes, 1985; Koronakis *et al.*, 1988a; Welch and Pellett, 1988). Information sufficient for membrane targeting and translocation of HlyA has been shown to be located in the C-terminal ~220 residues of the 1024-amino-acid protein. In the presence of HlyB and HlyD this region can direct apparently post-translational export of chimaeric protein into the bacterial growth medium (Nicaud *et al.*, 1986; Mackman *et al.*, 1987), supporting the view that the hemolysin secretion system might be open to biotechnological exploitation if further defined. The complete signal is apparently contained within the C-terminal 113 residues and it has been suggested that it may be located entirely within the extreme terminal 27 amino acid sequence (Mackman *et al.*, 1987). Nevertheless, the length and nature of the export signal remain undefined.

In this study data are presented which define the extent of the HlyA C-terminal signal directing wild-type hemolysin export into the surrounding medium and indicate that it has no primary structure requirement but has multiple secondary features. Identification of putative HlyA signal components was initiated by a comparison of homologous but divergent *hlyA* genes cloned from the related enterobacterial genera *Proteus* and *Morganella* (Koronakis *et al.*, 1987). Comparison of the three divergent HlyA C-terminal sequences revealed, in particular, the striking conservation of a potential amphiphilic α -helix immediately proximal to a cluster of charged residues and an extreme terminal weakly hydrophobic sequence rich in hydroxylated residues. We indicated the importance to export of these multiple protein sequences by internal in-frame deletion and progressive C-terminal truncation of the *E. coli* HlyA protein. In so doing we isolate for the first time the signal directing wild-type export as the extreme C-terminal 53 amino acids and show clearly by expression and export of a C-terminal peptide that it is not removed during translocation.

Fig. 1. The four contiguous genes of the enterobacterial hemolysin (*hly*) operon. Genes *hlyC* and *hlyA* determine the synthesis of intracellular active hemolysin, *hlyB* and *hlyD* dictate its complete export. The genes are transcribed coordinately with a site of transcription termination/antitermination in the 71-bp *hlyA*–*hlyB* intergenic space (Koronakis *et al.*, 1988a). Restriction nuclease sites used in the work were *Bgl*III (Bg), *Hpa*II (Hp), *Pst*I (P), *Eco*RI (R) and *Eco*RV (V). Numbers refer to base pairs of the *hlyA* sequence and the asterisks to restriction sites created by site-directed mutagenesis.

Results

HlyA C-terminal export sequences from three bacterial species

Production of cell-free hemolysin (HlyA) by *E. coli* is governed by four *hly* genes which dictate its synthesis (*hlyC* and *hlyA*) and export (*hlyB* and *hlyD*). The genes, which are contiguous and cotranscribed from upstream of *hlyC* (Koronakis and Hughes, 1988; Koronakis *et al.*, 1988a; Welch and Pellet, 1988), are depicted in Figure 1 together with the recombinant plasmid constructions used in the study. Previous work has demonstrated that the HlyB/HlyD-dependent export of hemolysin is directed by the C-terminal region of the 110-kD protein (Haertlein *et al.*, 1983; Mackman *et al.*, 1987). Experiments with chimaeric proteins have localized the secretion signal within the C-terminal 113 amino acids and it has been suggested that it may be contained entirely within the HlyA terminal 27 amino acids (Mackman *et al.*, 1987).

We have shown that *hly* genes of the enterobacterial species *Proteus vulgaris* and *Morganella morganii* are closely related to those of *E. coli* and that the hemolysin proteins of these species are exported normally by the *E. coli* *hlyB* and *hlyD* genes (Koronakis *et al.*, 1987). Nevertheless, the nucleotide sequences of the three *hly* operons show substantial sequence divergence (Koronakis *et al.*, 1987; Koronakis and Hughes, 1988). We exploited these relationships by isolating and sequencing the 3' *hlyA* sequences of *P. vulgaris* and *M. morganii* and comparing the potentially divergent HlyA C-terminal amino acid sequences with that of *E. coli* previously published (Hess *et al.*, 1986). The HlyA protein sequences compared were those encoded by the DNA sequence distal to the common *Eco*RV site at bp 2763 of the 3072-bp *hlyA* coding sequence (Figure 1) and were obtained from the plasmids pVU763–703 (*P. vulgaris*) and pMO227–208 (*M. morganii*) which have been previously described (Koronakis *et al.*, 1987). To demonstrate that these C-terminal 103 amino acid sequences (distal to residue 922) did indeed contain all the information needed for wild-type HlyA protein export, export was compared between the wild-type HlyA and a deleted derivative in which a 549-bp

Fig. 2. The C-terminal 103 amino acids determine wild-type levels of HlyA export. Extracellular HlyA protein exported by *E. coli* 5KC carrying pEK50X (wild type) or pEK50XΔV (in-frame *Eco*RV deletion within *hlyA*, Figure 1), stained with Coomassie blue.

in-frame deletion was created from the 2763-bp *Eco*RV site back to the *Eco*RV site at *hlyA* bp 2214 (Figure 1). Coomassie blue staining of the wild-type and deleted HlyA proteins exported into the cell-free extracellular medium showed that the deleted HlyAΔV protein directed levels of HlyB/HlyD-dependent export comparable to the wild-type HlyA (Figure 2). This export assay, like all others described in this work, was confirmed at more than one point during early- and mid-logarithmic growth.

The three deduced HlyA C-terminal sequences (distal to residue 922) are presented in Figure 3A. The possibility of identifying signal components conserved in the three proteins was greatly enhanced by the strikingly high level of amino acid substitution found throughout the sequences, which was in marked contrast to the closely conserved amino acid sequences observed in the HlyC and HlyB proteins (both >90%) (Koronakis and Hughes, 1988; Koronakis *et al.*, 1988b). The *Proteus* and *Morganella* 103-amino-acid C-terminal sequences differed from that of *E. coli* in 28 and 46 residues respectively, and from each other in 41 residues, indicating that extended primary protein sequence is not critical to the export signal.

Table I. The terminus of the export signal is weakly hydrophobic and rich in hydroxylated residues

	C-terminal residues				
	103–53	52–34	34–27	26–9	8–1
<i>E. coli</i>	–0.18/11.8%	+0.02/16.7%	–0.53/12.5%	–0.16/22.2%	+0.06/62.5%
<i>P. vulgaris</i>	–0.25/9.8%	+0.05/22.2%	–0.39/<12.5%	–0.06/33.3%	+0.15/50.0%
<i>M. organii</i>	–0.25/11.8%	–0.12/16.7%	–0.56/<11.7%	–0.02/44.4%	+0.22/37.5%

The mean hydrophobicity (Eisenberg *et al.*, 1982; positive numbers indicate hydrophobicity) and the percentage of hydroxylated residues (Ser and Thr) within the HlyA C-termini of the three proteins. Residues, numbered from the C-terminus, span the proposed features of the export signal (1–52) and the immediately proximal non-signal sequence (53–103).

Fig. 3. Conservation and divergence in the C-termini of three HlyA proteins (A) the 103 amino acid C-terminal sequences (residues 922–1024 distal to the corresponding conserved EcoRV site in *hlyA*) encoded by *E. coli* pANN202-312 (middle) (Hess *et al.*, 1986), *M. organii* pMO227-508 (lower) and *P. vulgaris* pVU763-710 (upper). Residues shared with *E. coli* are marked as points, charged residues are boxed and the potential amphiphilic α -helical sequence is underlined. The previously reported α -helix of *E. coli* (Mackman *et al.*, 1987) spans residues 990–1004 and within this sequence the additional Pro residue of *Morganella* HlyA is indicated by the vertical arrow (B) Conservation of the putative C-terminal amphiphilic α -helical sequence in the three HlyA proteins. The HlyA 18-residue sequences from 973 (numbered 1) to 990 in *E. coli* (outer), *M. organii* (middle) and *P. vulgaris* (inner). Points in the *Proteus* and *Morganella* sequences indicate identity to that of *E. coli*. Shaded residues are polar or charged, non-shaded are hydrophobic.

Conserved features of the HlyA C-terminus

The high background of amino acid substitution in the three proteins spotlighted a number of conserved features within the C-terminal half of the 103-residue sequence, specifically an extended extreme terminal hydrophobic sequence rich in

hydroxylated residues (residues 999–1024), a cluster of charged residues (residues 991–996, boxed in Figure 3) and a potential amphiphilic α -helix (residues 973–990, underlined in Figure 3).

The weakly hydrophobic sequence evident in the extreme C-terminal HlyA protein of *E. coli* was significantly more marked in both the *Proteus* and *Morganella* HlyA proteins. The mean hydrophobicity values of sequences within the three C-termini (Table I) show that in all three proteins the extreme terminal eight residue sequences were hydrophobic (mean hydrophobicity of *E. coli* +0.06, *P. vulgaris* +0.15 and *M. organii* +0.22). That this is a conserved feature is supported by the calculation that a second *E. coli* C-terminal sequence reported by Gray *et al.* (1986) yields a higher mean hydrophobicity of +0.11. The three C-terminal sequences between residues 1 and 26 from the terminus were in each case almost neutral in terms of hydrophobicity/hydrophilicity (mean hydrophobicity +0.05 (*M. organii*), +0.004 (*P. vulgaris*) and –0.09 (*E. coli*), particularly when compared with the HlyA sequence 53–103 residues from the C-terminus, i.e. proximal to the putative signal features. In addition to weak hydrophobicity, a striking feature of the C-terminal 26 amino acid sequences was the high incidence of hydroxylated residues; serine and threonine constituted in the three sequences 34.6% (*E. coli*), 38.5% (*P. vulgaris*) and 42.3% (*M. organii*) of residues.

At residues 991–996, boxed in Figure 3A, there is strong conservation of a block of highly charged sequence. Although there is frequent amino acid substitution, there is a pronounced maintenance of the charge sequence i.e. –0+ – – +. In the *Morganella* sequence the loss of negative charge caused by substitution of glutamic acid-995 with Thr appears to be specifically compensated by the unique substitution of alanine-999 to aspartic acid.

Immediately proximal to this charged cluster is a comparatively conserved primary sequence of 18 amino acids, between residues 973 and 990 (underlined in Figure 3A), which in all three HlyA proteins has the striking potential to form an amphiphilic α -helix, i.e. separating polar and hydrophobic residues on different helical faces. This potential is depicted by displaying the sequences on Schiffer–Edmunson helical wheels (Schiffer and Edmunson, 1967) (Figure 3B) which deploy residues at successive 100° turns, as they would be arranged along the axis of the amphiphilic α -helix. The charged/polar amino acids are clearly separated from the hydrophobic residues on opposite faces of the helix, and the length of the helix is in each case 18 residues, identical in length to those functional in, for example, membrane-bound bacterial penicillin-binding proteins (Jackson and Pratt, 1987) and mitochondrial porin (Kleene *et al.*, 1987). The presence of a proline at residue 3 of the 18-amino-acid sequence in *E. coli* may not be critical as shorter helices can

still be effective and indeed the well-characterized membrane-seeking peptide, mellitin, has a functional proline-kinked amphiphilic helix (Terwilliger *et al.*, 1982).

The relevance of a potential 15-amino-acid α -helix at residues 990–1004, previously speculated to be a possible export signal element in *E.coli* HlyA (Mackman *et al.*, 1987), is not lent support by the sequence comparisons, as the corresponding *Morganella* sequence has within it a radical substitution (of hydrophobic Ala to charged Asp) and, more significantly, an insertion of Pro, a residue prone to disrupt α -helices, at the mid-point of the proposed helical sequence. Indeed, computer secondary structure predictions (not shown; Garnier *et al.*, 1978) confirm that the *Morganella* sequence has lost entirely the region of strong α -helicity apparent in both *E.coli* and *P.vulgaris*.

The possible importance of the three conserved features described here, i.e. the amphiphilic α -helix, the charged cluster and the hydrophobic/hydroxylated tail was then investigated by examining export of engineered HlyA proteins in which the features had been independently deleted.

Deletion of the amphiphilic α -helix 34–52 residues from the C-terminus

To assess the biological significance of the potential amphiphilic α -helix, a pair of in-frame C-terminal deletions was made differing in the intact presence of the 18-amino-acid sequence at residues 973–990. This was achieved by independently fusing the N-terminal 830 residues of HlyA (not exported) to either the HlyA C-terminal 53 amino acids (to create plasmid pEK50X Δ Bg53) or the C-terminal 38 amino acids (pEK50X Δ Bg38); specifically by ligating the 5' bulk of the *hlyA* gene, truncated at the native *Bgl*III 2491 restriction site (Figure 1), to the 3' *hlyA* sequence isolated by either of two new *Bgl*III sites created independently by oligonucleotide-directed mutations in pEK50X. The Bg53 restriction site was achieved in one mutational step with oligonucleotide 530, while restoration of the *hlyA* reading frame distal to the new Bg38 site required a second insertional mutation (i.e. oligonucleotide 380 followed by 381, see Materials and methods). The new amino acid sequences generated at the two fusion points are shown in Figure 4A.

Complementation of the two deleted plasmids pEK50X Δ Bg53 and pEK50X Δ Bg38 with the plasmid pLG575, which expresses the secretion genes *hlyB* and *hlyD*, allowed direct assessment of *E.coli* 5KC export of the resulting 883- and 868-residue HlyA fusion proteins into the surrounding medium. Figure 5A shows that pEK50X Δ Bg53 directed *hlyB/hlyD*-dependent export at a level comparable to that level determined by the wild-type pEK50X, but export directed by pEK50X Δ Bg38 was reduced by ~90%. These results are compatible with the presence of elements critical for secretion in the terminal 27 (Mackman *et al.*, 1987) or 37 amino acids (note added in proof Mackman *et al.*, 1987) but show that these elements can direct only low levels of export. Wild-type export requires the presence of the immediately proximal sequence determining the amphiphilic α -helix. The data describe therefore the first isolation of the export signal directing wild-type HlyA export into the medium, i.e. the C-terminal 53 amino acids.

Fig. 4. (A) In-frame deletion of *hlyA* to (i) eliminate or retain the potential amphiphilic sequence by respective fusion of HlyA' to the C-terminal 38 residues (pEK50X Δ Bg38) or 53 residues (pEK50X Δ Bg53), and (ii) eliminate the cluster of charged residues (pEK50X Δ P). The amino acid sequences were created at the three fusion points. (B) Recreation and extension of the potential amphiphilic helix in pEK50X Δ P; shaded residues are polar or charged, non-shaded residues are hydrophobic.

Fig. 5. HlyA protein exported into the medium by *E.coli* 5KC carrying (A) pEK50X, pEK50X Δ Bg53 and pEK50X Δ Bg38 and (B) pEK50 and pEK50X Δ P; all stained with Coomassie blue.

Deletion of the charged cluster 29–34 residues from the C-terminus

The cluster of negatively and positively charged residues (991–996) immediately distal to the proposed amphiphilic α -helix is conserved in all three HlyA proteins despite a high incidence of substitution of the individual residues. To remove the charged cluster while retaining the amphiphilic helix and the hydrophobic tail an in-frame deletion of *hlyA* codons 988–998 was made using the native *Pst*I sites at these codons (see Materials and methods) yielding the fusion

Fig. 6. Effect of progressive truncation of the HlyA C-terminus. (A) Location of mutant translation stop codons introduced into *hlyA* of pEK50X (all six mutations) and pEK50XΔBg53 (the three extreme terminal mutations). Charged residues are boxed, the amphiphilic α -helical sequence is underlined and the mutational substitutions made at the numbered codons are shown above the native sequence. Export of HlyA' protein by *E. coli* 5K carrying (B) wild-type and three progressively truncated 3' *hlyA* sequences in pEK50XΔBg53 and (C) wild-type and six progressively truncated 3' *hlyA* sequences in pEK50X.

sequence shown in Figure 4A. The deletion of only 11 amino acids not only retained the full distal hydrophobic/hydroxylated sequence but also recreates almost perfectly the proximal amphiphilic sequence, indeed it extends its potential length to 21 residues (Figure 4B). The assay of cell-free extracellular HlyA protein revealed that the independent deletion of the charged cluster in pEK50ΔP had caused a virtual elimination of export (Figure 5B). It also demonstrated clearly that the terminal 27 amino acids are incapable of directing significant export alone (the *PstI* deletion leaves the terminal 26 residues and completes the 27-amino-acid sequence by replacing the Ala-27 residues from the terminus with the Ala encoded at the proximal *PstI* site).

Deletion of the extreme C-terminal hydrophobic/hydroxylated sequence

Our comparative sequence data indicated that the prominent features of the extreme C-terminal protein were not primary sequence conservation but rather weak hydrophobicity, clear within the eight extreme terminal residues but extending less prominently up to 26 residues into the protein, and an

extremely high incidence of hydroxylated residues. To assess the possible importance of the hydrophobic/hydroxylated tail, we introduced three translation stop codons, shown in Figure 6A, to progressively truncate by 8, 21 and 27 residues the isolated, export-competent 53-residue C-terminal signal of pEK50XΔBg53. Comparative assay of the extracellular truncated HlyA proteins (Figure 6B) showed that mutation 1017, which removed the extreme terminal eight-residue sequence (hydrophobic in each of the three HlyA proteins) and created a hydrophilic tail (the new terminal six amino acids have a mean hydrophobicity of -0.32) caused a moderate ($\sim 50\%$) decrease in export. In contrast, the removal by mutation 1004 of the C-terminal 21 residues, which restores a hydrophobic tail (mean hydrophobicity of the six new terminal residues $+0.27$) radically reduced HlyA export into the medium, indeed virtually no HlyA protein was visible in the cell-free medium. This very low level was not increased when this remaining six-residue hydrophobic sequence was removed by mutation 998 making the extreme C-terminus very strongly charged. The effect of these three truncations in the isolated C-terminal signal of

pEK50XΔBg53 were substantiated by a parallel introduction of all six mutant translation stop codons into the full undeleted *hlyA* sequence of pEK50X. Figure 6C shows the uneven progressive impairment of export caused by the six increasing truncations of the C-terminal 53 residues. As seen with the pEK50XΔBg53 mutants, while reduction of the tail by mutation 1017 caused a moderate reduction in HlyA export of ~30–50%, further truncations caused radical impairment. The low levels of extracellular HlyA achieved following truncation by mutations 1004 and 998 (<5% and virtually identical) were seen more clearly in this series of assays and they were visibly reduced still further, essentially to zero, when the amphiphilic and charged regions were removed or disrupted by mutations 985, 982 and 976.

Impairment of the C-terminal export signal causes HlyA accumulation intracellularly but not in the periplasm

Hemolysin generates membrane-damaging pores in a range of mammalian cells, of normal tissue and the immune system, and it has been suggested that a hydrophilic–amphiphilic transition may be critical to targetting into eukaryotic membranes (Bhakdi *et al.*, 1986). Variant HlyA proteins were assayed for their hemolytic activity by introducing mutant pEK50 and pEK50X plasmids into *E.coli* 5KC lacking HlyB and HlyD and measuring intracellular activity. None of the deletions and truncations made in HlyA, including those eliminating the C-terminal amphiphilic sequence, caused a significant change in activity (Table II). The C-terminal amphiphilicity is thus not central to the eukaryotic membrane-damaging function of HlyA and the results also support the view that the alterations in the C-terminus had no secondary effects on the hemolytic activity or stability of the mature HlyA protein. In the presence of HlyB and HlyD, the HlyA variants impaired in export were moderately accumulated within the cell during late-logarithmic phase [even when export is at wild-type levels there is a pool of intracellular activity and accumulation is typically undramatic in export variants defective in HlyA or HlyB (Koronakis *et al.*, 1988b)]. Parallel hemolytic activities of whole cultures or resuspended washed cells (Table II) showed that in no case where cell-free activity

was lost was the non-exported activity cell-associated, i.e. bound to the outer membrane but not released (Koronakis *et al.*, 1987).

To confirm the above results and expand them to the extensively deleted HlyA proteins not retaining hemolytic activity, i.e. pEK50XΔBg38 and those of the pEK50XΔBg53 series, immunoblots were performed on fractions of *E.coli* cells carrying HlyB and HlyD (Figure 7). Antiserum raised against denatured extracellular HlyA identified intracellular variant HlyA protein whether hemolytically active or inactive [the latter variants less so, presumably due to the loss of the extensive repeat structures described ~200–300 residues from the C-terminus (Hess *et al.*, 1986)]. This gain suggests that export mutations caused no significant secondary effect on protein stability and this was still further emphasized by confirmation of unchanged stabilities of HlyA protein and hemolytic activity in extracellular supernatant (not shown). Fractionation of the cells indicated again the moderate accumulation of export-defective HlyA in the cytoplasm, but in no case, wild-type or export-defective, was

Table II. Hemolytic activities of *E.coli* 5KC synthesizing wild-type or mutant HlyA

	Cell-free	Whole culture	Intracellular	
			– B/D	+ B/D
HlyA ^a	210.5	227.8	–	63.4
HlyAΔP ^a	18.7	22.2	–	131.9
HlyA	217.3	210.4	130.6	59.2
HlyA 976	0.5	0.6	135.7	144.3
HlyA 982	0.3	0.5	121.7	142.3
HlyA 985	0.7	0.7	144.1	131.8
HlyA 998	3.1	3.9	139.1	110.0
HlyA 1004	4.5	8.6	128.2	113.4
HlyA 1017	72.1	82.3	132.8	78.9

Activities were measured in late-logarithmic growth ($A_{600} 0.75 \pm 0.5$), tared against activity determined by the extensively deleted HlyA of pEK50ΔV, and expressed in hemolytic units. Whole culture activities were reproduced to within 10% when cells were harvested, washed and resuspended in medium. The *hlyA* genes of pEK50 and pEK50ΔP (marked ^a) are contiguous with the export genes *hlyB* and *hlyD*; all the remainder, of the pEK50X series, are on separate compatible plasmids.

Fig. 7. Western blot analysis of HlyA protein present within *E.coli* 5KC carrying wild-type and 3' deleted and truncated *hlyA* genes. Protein from each bacterial culture was assayed in periplasmic (P), membrane (M) and cytoplasmic (C) fractions and blotted with rabbit antiserum raised against exported cell-free exported HlyA.

HlyA found in the periplasmic fraction. The levels of HlyA in the membrane fraction indicate either a residual signal-independent membrane association of export-defective HlyA protein or non-specific association during fractionation.

The HlyA C-terminal signal is not removed during export

Our data indicate that the information required for full export of hemolysin protein is carried in the C-terminal 53 amino acids. Assessment of whether export involves cleavage of this C-terminal signal would not be feasible in the complete 110-kD HlyA protein as small truncations would not be visible (see for example Figure 6B), so a short export-competent C-terminal peptide (CTP) containing the signal was compared in its intracellular and extracellular forms. The HlyA C-terminal 194 amino acid sequence was synthesized as a 205-amino-acid fusion peptide following the induction of specific expression in mid-logarithmic phase cultures of *E. coli* carrying the 3' *hlyA* sequence cloned into a T7 bacteriophage expression vector (Figure 8A and Materials and methods). The CTP was synthesized at high levels, remaining stable intracellularly, and when complemented with the export proteins HlyB and HlyD (encoded by plasmid pLG575) it was exported readily into the medium. Electrophoresis and staining of the denatured intracellular and exported CTPs (Figure 8B) revealed no discernible reduction in size between the intracellular and extracellular forms. This was emphasized by comparison with an intracellular non-exported 151-residue peptide lacking the extreme C-terminal 39 amino acids, again stable intracellularly, which was generated in parallel to the truncated 985 HlyA C-terminal sequence.

Discussion

We present here comparative and mutational analyses which define the extent of the HlyA C-terminal signal directing wild-type export of the hemolysin protein into the surrounding medium. We have confined our manipulations of the *E. coli* C-terminus to truncations and in-frame deletions of the HlyA protein itself in order to avoid potentially misleading effects arising from heterologous fusions. In this way it was demonstrated that the final *E. coli* 103 C-terminal residues can direct transport of the HlyA protein at wild-type levels. Comparison of this 103 residue *E. coli* sequence with the functionally identical but remarkably divergent

C-terminal sequences of two other enterobacteria, *P. vulgaris* and *M. morganii*, then highlighted shared features of the export signal sequence. The high levels of amino acid substitution (27, 40 and 45%) between the closely related proteins indicated a lack of extended primary sequence requirement in the signal but highlighted within the terminal 53-amino-acid sequences three conserved secondary features. Firstly, the extreme C-terminal eight residue sequences are hydrophobic, weakly so up to ~26 residues into the protein, and are extremely rich in hydroxylated residues. Secondly, proximal to this terminal hydrophobic/hydroxylated sequence, 29–34 residues from the terminus, is a strongly charged cluster of acidic and basic charged residues, and thirdly, immediately proximal to both these two features, at residues 35–52 from the HlyA C-terminus, is a potential 18-amino-acid amphiphilic α -helix.

The three conserved features were deleted independently in the *E. coli* HlyA protein by internal in-frame deletion, or progressive C-terminal truncation engineered by the introduction of translation stop codons into the *hlyA* 3' sequence. Removal of the hydrophobic eight-amino-acid sequence at the extreme C-terminus, leaving a hydrophilic terminus, caused a clear but nevertheless moderate impairment of export, by ~50–70%. Truncation of the C-terminal 21 residues, which deleted practically all the hydrophobic/hydroxylated tail but restored a clearly hydrophobic extreme terminus, reduced drastically the export of HlyA, to well below 5%. This low level was unaltered by the removal of six further residues, i.e. the C-terminal 27 residues, which removed all the tail and exposed an extremely charged extreme terminus. These data confirm previous indications (Mackman *et al.*, 1987) that the C-terminal tail of 26/27 residues is essential for export, and show that this is equally true of the C-terminal 21 residues. They show that the eight-residue extreme terminal hydrophobic/hydroxylated sequence is a contributory but not critical element and therefore indicate that an as yet undefined property of the 8–21 terminal residues is of particular importance. Computer secondary structure prediction (Garnier *et al.*, 1978) indicates no helical potential in the tail. Deletion of the cluster of charged residues 29–34 residues from the terminus by an 11-amino-acid deletion within the HlyA terminus resulted in the total elimination of export despite the C-terminus retaining both the hydrophobic/hydroxylated tail and the upstream potential amphiphilic α -helix. The charged cluster, which conserves the

Fig. 8. (A) Expression of the 194-amino-acid HlyA C-terminal sequence as a 205-residue fusion peptide. (B) Comparison of intracellular (in) and exported (out) C-terminal fusion peptide (CTP) generated from T7 expression plasmid pAR30. The 39-residue-shorter intracellular peptide (CTP39) was generated in parallel from truncated *hlyA* A985 sequence. Intracellular preparations were made from cells lacking *hlyB* and *hlyD*.

charge distribution $-0+--+$ but not primary sequence, has previously been speculated (Mackman *et al.*, 1987) to form the N-terminal part of an α -helix located ~ 20 – 34 residues from the C-terminus, and if this is the case then the in-frame deletion could have eliminated the export as a result of the loss of this helix. Nevertheless our finding that the *Morganella* HlyA sequence has an insertion of a Pro residue immediately distal to the charged cluster and at the very centre of the previously proposed helix supports the likelihood that the deleterious deletion owes its effect to the loss of the charged residues *per se*.

Removal of the third putative signal feature, the potential amphiphilic α -helix located 35–52 residues from the terminus, was achieved by fusing the 38 C-terminal residues to the N-terminal 830 amino acids of HlyA and this deletion caused a dramatic, $\sim 90\%$, reduction in the extracellular level of protein. In contrast, a parallel fusion of the same N-terminal HlyA protein to the C-terminal 53 residues, the minimum sequence bearing all three conserved features, directed HlyA export at levels indistinguishable from the wild-type. Signal loss caused typically moderate accumulation of HlyA intracellularly but export-defective HlyA proteins were evidently not blocked in the periplasm supporting the thesis that HlyA is exported directly into the media via a HlyB/HlyD channel without a periplasmic intermediate. Immunoblotting and hemolysin assays indicated that C-terminal manipulations had no secondary effect on HlyA hemolytic function or stability.

The analysis of exported and intracellular C-terminal HlyA variants shows therefore that the HlyA export signal extends 52/53 amino acids into the C-terminus, that it requires the three conserved features in concert for full export across both membranes, and that it can function independently of the HlyA protein. The independent export of the extreme C-terminal 194 amino acids as a 205-residue peptide supports this last point and comparison of the intracellular and secreted extracellular forms of the peptide showed that neither the 53-residue sequence nor its 26-residue terminus is removed during translocation. The isolation of the complete HlyA C-terminal signal opens further the way for the possible efficient export of recombinant proteins across both membranes out of the bacterial cell, and provides a parallel to the recent isolation of the 37-amino-acid C-terminal signal of eukaryotic decay acceleration factor (DAF) which can be used to target fusion proteins into the surface membrane (Caras *et al.*, 1987).

Are there analogies between the emerging nature of the HlyA C-terminal signal and other translocation signals or is the hemolysin system really unique? It would indeed be surprising if, at a time when the common features of cotranslational and post-translational translocation are becoming increasingly evident (Rothman and Kornberg, 1986; Schatz, 1986), the hemolysin export was based on unique features. It is therefore not unexpected that the putative signal features highlighted here are reminiscent of other prokaryotic and eukaryotic membrane translocation signals. First of all the lack of primary sequence homology between the three functionally homologous HlyA C-termini indicates that, analogous to N-terminal secretion and mitochondrial import signals (Michaelis and Beckwith, 1982; Bankiatis *et al.*, 1984; Allison and Schatz, 1986), the transport process is addressed not by primary sequence consensus but by secondary features. In particular, the

proposed involvement of the amphiphilic α -helix secondary structure, typical of peptide sequences interacting with phospholipid membranes, e.g. as essential components of the receptor binding domain of glycoprotein hormones (Keutmann *et al.*, 1987) and the C-terminal membrane anchoring domains of *E. coli* penicillin-binding proteins (Jackson and Pratt, 1987), provokes a possible analogy with mitochondrial import pre-sequences. These N-terminal cleaved sequences target proteins to the mitochondrial matrix, and have the ability to form amphiphilic α -helices (Epand *et al.*, 1986; Roise *et al.*, 1986; von Heijne, 1986), structures which can insert directly into phospholipid bilayers and which therefore could be critical to initiating protein association with the membrane or the transfer of protein by responding to transmembrane potential. Recent analysis of artificial pre-sequences has suggested that amphiphilicity alone, in the absence of helicity, may be the critical feature (Roise *et al.*, 1988) and by extension this may be possible in HlyA. Interestingly, when considering the putative features of the hemolysin signal, the mitochondrial amphiphilic secondary structures are also seen to be associated with charged residues, which may be important to provide a driving force for pre-sequence translocation (Roise *et al.*, 1988), and to be rich in hydroxylated residues (Roise *et al.*, 1986; von Heijne, 1986). The HlyA C-terminal signal can be exported independently as a short C-terminal peptide and can direct heterologous transport; its loss or impairment does not affect the hemolytic activity or stability of the HlyA protein. These observations again suggest analogy with mitochondrial pre-sequences, which can achieve import of heterologous protein while maintaining folding and action independent of the mature protein (Eilers and Schatz, 1986).

Possible analogies between the terminal signals of bacterial hemolysin and mitochondrial proteins are especially enticing as mitochondrial import also proceeds across two membranes and apparently requires ATP, transmembrane potential and a proteinaceous signal sequence receptor (Zwizinski *et al.*, 1984). The hemolysin export is governed by two specific membrane proteins HlyB and HlyD, suggested to cooperate in creating a pore spanning both membranes. HlyB shares with bacterial and mammalian transporters both potential membrane-spanning regions and ATP-binding domains indicating it may energize the membrane translocation process (Higgins *et al.*, 1986; Gerlach *et al.*, 1986) and although HlyD remains as yet anonymous one possible role would be as a membrane receptor for the C-terminal signal of HlyA. With the components of the hemolysin system already isolated and mutants available, further study may provide results of wide relevance to protein translocation.

Materials and methods

Plasmids

The recombinant plasmid pEK50 is pBR322 based and bears the entire fully active four-gene *hly* determinant of *E. coli* pHly152 (Hess *et al.*, 1986; Koronakis *et al.*, 1988a; pEK50X (carrying only the synthesis genes *hlyC* and *hlyA*) was derived from this plasmid by *in vitro* deletion of the second half of the *hly* determinant (from the *EcoRV* site 285 bp into *hlyB* to the vector *PvuII* site distal to *hlyD*). The plasmid pEK50 Δ P was constructed by initial partial *PstI* deletion within the cloned *BglII* fragment [from *BglII* 2491 in the *hlyA* 3' terminus to the *BglII* site distal to *hlyD* (Hess *et al.*, 1986)] before reintroduction of the *PstI*-deleted *BglII* fragment into the full *hly* determinant of pEK50. Other mutated derivatives of pEK50X were constructed by deletion and truncation of the *hlyA* 3' terminus using both restriction sites and translation stop codons created by site-directed

Fig. 9. (A) In-frame deletion of pEK50X 3' *hlyA* retaining and removing the proposed C-terminal amphiphilic α -helix sequence (underlined) in plasmids pEK50XΔBg53 and pEK50XΔBg38 respectively, and retaining and removing the cluster of charged residues (boxed) in pEK50 and pEK50ΔP. (B) Introduction of site-directed mutations into the functional *hly* determinant. Mutations destined for pEK50X and pEK50XΔBg53 were introduced respectively into *hlyA* 3' within *EcoRV* and *EcoRI*-*HpaI* restriction fragments cloned into M13mp9 *SmaI* and M13mp9 *EcoRI*-*SmaI*. Mutated sequences, shown as closed squares, were then reintroduced into the functional *hly* plasmids as *BglII*-*BamHI* fragments into the *BglII* sites of *hlyA* and *hlyB*. Restriction nuclease sites are *BamHI* (B), *BglII* (Bg), *HpaI* (Hp), *PstI* (P), *EcoRI* (R), *SmaI* (S) and *EcoRV* (V).

mutagenesis. The details of these constructions are depicted in Figure 9A and B and were all confirmed by sequencing and restriction nuclease mapping. Plasmid pLG575 is pACYC184 based and expresses the *E. coli* secretion genes *hlyB* and *hlyD* (Mackman *et al.*, 1985). All *hly* plasmids were carried in *E. coli* 5KC (*recA1*, *hsdR*, *hsdD*).

DNA sequencing and oligonucleotide-directed mutagenesis

The nucleotide sequences of the *P. vulgaris* and *M. Morganii* *hlyA* genes were derived from the previously described recombinant DNAs pVU763-710 and pMO227-508 bearing the *hly* determinants of the clinical isolates *P. vulgaris* 76362 and *M. Morganii* 227 (Koronakis *et al.*, 1987). The 3' *hlyA* nucleotide sequence was determined from both strands by the Sanger dideoxy method from recombinant M13 phage (Sanger *et al.*, 1977) or directly by the method of Maxam and Gilbert (1980). Mutagenic oligonucleotides (listed in Table III) were synthesized by the phosphoramidite method on an Applied Biosystems Synthesizer and purified by 7 M urea/20% polyacrylamide gel electrophoresis (PAGE). Site-specific mutagenesis was performed by the double-primer approach (Kramer *et al.*, 1984) using phage M13mp10. Single-stranded template (1 µg) was annealed with 10 pmol of phosphorylated mutagenic primer and 10 pmol of phosphorylated M13 universal primer in 10 µl of 10 mM Tris-Cl, 10 mM MgCl₂, pH 8.0 before primer extension by 1 unit of DNA polymerase Klenow fragment for 4 h at 12°C in the presence of 0.2 mM dNTPs, 0.25 mM ATP and 5 units of T4 ligase (all from Boehringer). The mutagenized double-stranded DNA was then used to transfect *E. coli* strain TG1 and transformants carrying the mutant sequence were identified by plaque hybridization with the mutagenic oligonucleotides. Mutant DNA was isolated after retransfection and all nucleotide changes were confirmed by sequencing. Confirmed mutations were introduced into functional *hlyA* genes either in pEK50 or pEK50X.

Assay of hemolysin (HlyA) protein export

The extracellular presence of the previously well-characterized 100-kD HlyA protein in the above sample was assayed by concentrating 20 ml of cell-free medium by TCA (10% v/v, 0°C) precipitation. Proteins were redissolved in 100 µl SDS loading buffer, neutralized with Tris base and heated in boiling water for 10 min before separation on a 15% acrylamide gel. Gels were stained with Coomassie blue.

Hemolytic activity was measured during batch growth of *E. coli* 5KC transformants at 37°C, shaking in Brain Heart Infusion broth containing 50 µg/ml ampicillin. Aliquots of culture were removed at intervals during exponential growth (A_{600} 0.25–0.85) and assayed for cell-free secreted hemolytic activity in the supernatant following centrifugation. Parallel intracellular and cell-bound hemolytic activities were assayed respectively, in cleared cell extracts of bacterial cells lysed by lysozyme treatment and whole cultures (Koronakis *et al.*, 1987, 1988b).

Cell fractionation

Fractionation of mid–late-logarithmic growth phase *E. coli* 5KC was achieved essentially as described by Manoel and Beckwith (1986). Centrifuged cell pellets from 15 ml culture (A_{600} 0.65–0.75) were resuspended in 100 µl of cold spheroplast buffer (SB, 100 mM Tris-Cl, pH 8.0, 0.5 mM EDTA, 0.5 M sucrose) containing 20 µg/ml of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) and incubated for 5 min on ice. Following centrifugation (3 min, 15 000 g, 4°C) and resuspension the cells were osmotically shocked in 100 µl cold sterile distilled water, to which was added 5 µg/ml 20 mM MgCl₂ after 45 s. Centrifugation as above yielded the periplasmic fraction as supernatant. The pellet was resuspended in 150 µl cold SB to which was added 15 µl 2 mg/ml lysozyme and a further 150 µl SB; incubation for 5 min on ice was followed by centrifugation as above. The pellet was resuspended in 600 µl Tris-Cl, pH 8.0 containing 20 µg/ml PMSF and frozen and thawed three times to produce lysed spheroplasts prior to adding 20 µl 1 M MgCl₂ and 6 µl of 1 mg/ml DNase I. These

were then centrifuged for 25 min as above to yield the membrane fraction (pellet), and the cytoplasmic fraction (supernatant 5-fold concentrated with TCA). Total protein profiles of each fraction were controlled qualitatively by SDS–PAGE.

Western blotting

Proteins present in the whole cell, periplasmic, membrane and cytoplasmic fractions were separated by electrophoresis through 12.5% polyacrylamide gels containing 10% SDS and transferred to nitrocellulose paper which was subsequently blocked (0.01 M Tris, 0.15 M NaCl, pH 7.4, 3% BSA). Recognition by rabbit antiserum (raised against denatured extracellular HlyA) protein was achieved (Towbin *et al.*, 1979) by incubation at 20°C for 1 h at a 2500 dilution in Tween buffer (0.01 M Tris, 0.15 M NaCl, pH 7.4, 0.05% Tween). Following 3 × 7-min washes in Tween buffer bound rabbit antibody was incubated for 1 h at 20°C with donkey anti-rabbit immunoglobulin (1:500 dilution in Tween buffer). Following three further washes in the same buffer the biotin/streptavidin/peroxidase complex (1:400 dilution in Tween buffer) was added for 30 min at 20°C. After further washing the nitrocellulose was bathed in fresh cold substrate (60 µl peroxide, 60 mg chloronaphthol, 20 ml methanol in 60 ml phosphate-buffered saline) and developed.

Synthesis and export of the HlyA C-terminal peptide

The HlyA C-terminal 194 amino acid sequence was generated as a 205-residue fusion peptide from the plasmid pAR3039 (Studier and Moffatt, 1986) carrying the native *hly* BgIII restriction fragment (the 5' end of which is bp-2491 of *hlyA*, Figure 1). The fragment was cloned downstream of the T4 phage promoter, the ribosome-binding site, and in-frame with the initial 11 codons of T7 gene 10 (Figure 8A). Specific high-level expression from the T7 promoter followed induction of *lac* UV5-directed transcription of the chromosomal T7 RNA polymerase gene 1 of lysogen *E. coli* BL21(DE3) cultures (A_{600} 0.5) with 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells and supernatant were collected after 3 h continuous incubation. Intracellular peptide was identified in cells lacking *hlyB/hlyD* after boiling cells in SDS loading buffer, extracellular peptide was assayed in cells expressing both *hlyB* and *hlyD* after TCA precipitation of culture supernatant, by Coomassie blue staining.

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Table III. Mutagenic oligonucleotide sequences (5'–3')

	Sequence
976	– T T A A T C C A T A A A T T A A T G –
982	– G A A A T C A G C T A A A T C A T T –
985	– A A A T C A T T T A A G C T G C A G –
998	– G A A A G A A C T T A A G C T T C T T T –
1004	– T A T T G C A G T A G T C C G G T A –
1017	– A C G G A A C T A A A T A A C C C T –
380	– T C A T T T C A G A T C T G C A G G T –
381	– T T C A G A T C T G C A G G T A –
530	– A A G T C A G G G A G A T C T T A –

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