The C-terminal, 23 kDa peptide of *E. coli* haemolysin 2001 contains all the information necessary for its secretion by the haemolysin (Hly) export machinery

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In this paper we show the construction of a plasmid pLG609 which carries the 3'-end of the haemolysin structural gene, *hly*A under *tac* promoter control. Expression of pLG609 in an *E. coli* strain carrying the haemolysin export genes *hly*B and *hly*D led to the efficient secretion of the C-terminal, 23 kDa peptide of haemolysin. The discovery of a C-terminal topogenic sequence, which appears to be all that is required for secretion of the whole toxin, is so far quite unique in protein export.

(E. coli) *Hemolysin* *C-terminal secretion signal* *Expression vector* *Protein export*

1. INTRODUCTION

The synthesis and export of polypeptides across membranes is normally a tightly coupled process and is remarkably conserved throughout nature, being almost invariably dependent upon a hydrophobic signal sequence present at the N-terminus of the exported polypeptide [1,2]. This signal facilitates the initial interaction with membranes, promoting translocation of the protein and then cleavage of the signal which releases the exported polypeptide [3]. Unfortunately, the details of the precise mechanism of the translocation event are obscure. Moreover, the additional signals necessary for directing many exported polypeptides to specific locations, following their initial transport, are also unknown. We are investigating some of these questions with the *Escherichia coli* haemolysin system. This involves the export of a 107 kDa polypeptide through both the inner and the outer membrane to the external medium. In this paper we show that all the information necessary for recognition and export of the toxin, via Hly proteins B and D, is apparently contained within the last (C-terminal) 23 kDa of the molecule.

The production of haemolysin by certain pathogenic strains of *E. coli* is encoded by an approx. 7.5 kilobase sequence present on plasmids or integrated into the chromosome [4,5]. The Hly determinant is composed of at least 4 genes: *hly*A, the structural gene for the toxin; *hly*C, required for some form of post-translational modification of HlyA but not for its secretion and *hly*B *hly*D, which are both essential for export of the toxin through the envelope and into the medium [6–8].

Previous studies have demonstrated that *hly*A encodes a 107 kDa polypeptide [9,10] which is exported to the medium without proteolytic processing of the N-terminus [11]. Furthermore, DNA sequence data for *hly*A indicates the complete absence of an N-terminal signal sequence [12]. In view of this and the apparent absence of a periplasmic intermediate in haemolysin secretion [13], we have proposed a model for export of the haemolysin [13]. We envisage a first step involving

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recognition of cytoplasmic HlyA by the export machinery located in the inner membrane followed by a second step which results in direct extrusion of the toxin to the medium.

2. MATERIALS AND METHODS

2.1. *Strains and plasmids*

The laboratory *E. coli* K12 strain SE5000 *rps*L150, *ara*139, Δ(*lac*IPOZYA) U169, *rec*1A, *pts*F25, *flb*301, *deo*C1, *rec*A57 was used in all these studies. The plasmid pLG570 contains the haemolytic determinant from the wild-type *E. coli* strain LE2001 isolated as described in [14]. The plasmids pLG575 and pLG579, which carry either the complete export functions *hly*B + *hly*D or only *hly*B, respectively, have been described elsewhere [7]. Plasmid pTTQ18 was kindly provided by Dr Mike Stark (Leicester Biocentre) and is a derivative of pUC18 carrying the $lacI^q$ gene to ensure maximum repression of the *tac* promoter in this high (80) copy plasmid. Full details of its construction will be published at a later date.

2.2. *Media*

Cultures were grown in rich medium (Nutrient broth – Oxoid) containing the appropriate antibiotics used at the following concentrations – 10 μg/ml tetracycline and 25 μg/ml ampicillin/chloramphenicol.

2.3. *Construction of plasmid pLG609*

Plasmid pLG579 [7] was digested with *Eco*RI and *Hin*dIII and a 1.6 kb fragment was purified from an agarose gel after electrophoresis [15]. This fragment was then ligated into the *Eco*RI-*Hin*dIII sites contained in a multi-cloning sequence of the expression vector pTTQ18 to form the recombinant plasmid pLG609. This plasmid contains the 3′-terminal region of *hly*A and the 5′-region of *hly*B downstream from an inducible *tac* promoter. The *hly*B sequence is followed by those encoding the α-peptide of β-galactosidase [16] and a transcriptional terminator, $rrnBt_1t_2$.

2.4. *Preparation of protein samples*

Cultures were grown in nutrient broth and at A_{450} = 0.5 *o*-nitrophenyl-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM in order to induce transcription from the *tac* promoter. 20 ml samples were harvested by centrifugation (Sorvall 18K, 15 min, SS34 rotor) and trichloroacetic acid added to the supernatant to 10% final concentration. After 30 min incubation on ice, proteins were sedimented by centrifugation (Sorvall 10K, 15 min, HB4 rotor). The protein pellet was then resuspended in SDS-sample buffer and a concentrated Tris solution was used to restore pH 6.8.

2.5. *Intracellular and extracellular detection of the 107 and 23 kDa polypeptides using antibody*

As described [17] proteins were separated by SDS-PAGE (15% acrylamide) and then transferred to nitrocellulose by Western blotting. The 107 and 23 kDa proteins were immunologically detected using anti-haemolysin antibody followed by peroxidase-conjugated antibody.

3. RESULTS

3.1. *Subcloning the 3′-end of HlyA under* tac *promoter control*

As the *E. coli* haemolysin had been previously shown not to possess an N-terminal signal sequence [11], we have investigated the possibility that information required for secretion of HlyA might be entirely located in the C-terminus of the protein. This was achieved (see fig.1) by subcloning a 1.6 kb *Eco*RI-*Hin*dIII fragment encoding the 3′-end of *hly*A, in the correct reading frame for subsequent translation, into the high copy expression vector, pTTQ18. This plasmid carries a *tac* promoter upstream of a multicloning region, together with the $lacI^q$ gene in order to ensure that expression from the cloned gene is completely repressed in the absence of the inducer IPTG. The resulting recombinant plasmid, pLG609, was expected to contain a hybrid gene with the *tac* promoter fused to the 3′-end of *hly*A which should direct the synthesis of a 23 kDa polypeptide (Ac) corresponding to the C-terminus of HlyA. The presence and orientation of the *Eco*RI-*Hin*dIII fragment in the plasmid vector was confirmed by restriction enzyme analysis.

3.2. *Growth characteristics of a strain carrying pLG609*

The growth characteristics of strain SE5000

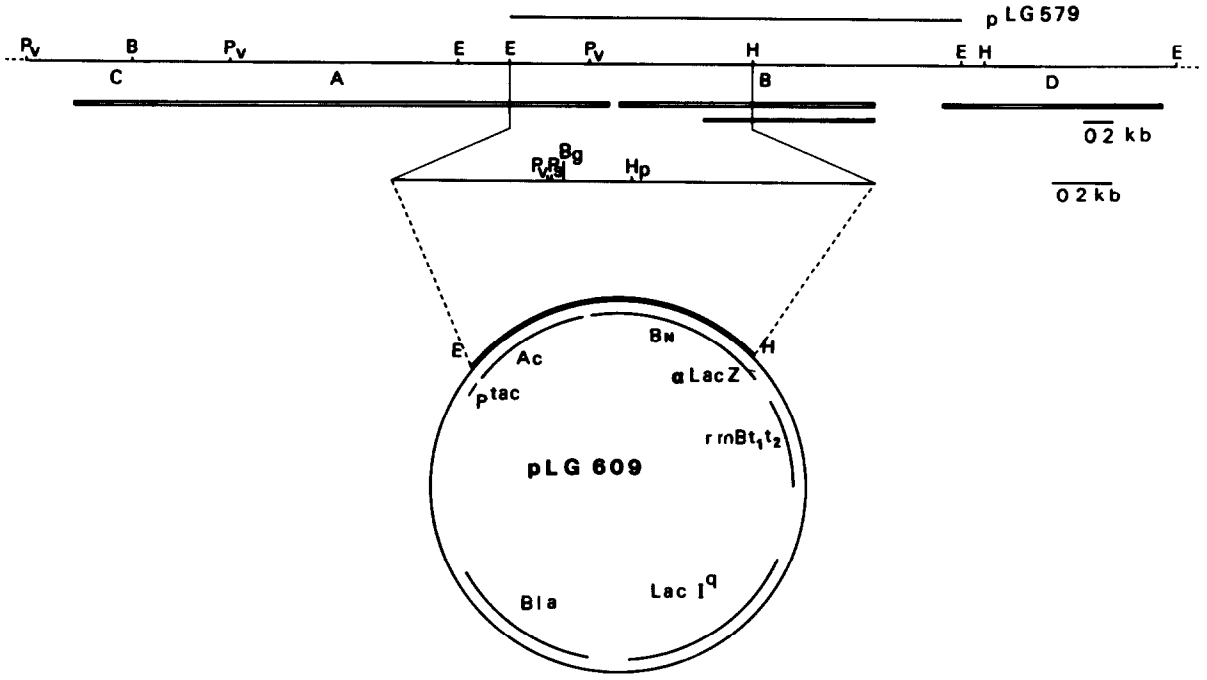


Fig.1. A restriction map of a portion of the recombinant plasmid pLG570 is presented. This plasmid contains the Hly determinant 2001 [7] and encodes the polypeptide products of *hly*C, A, B and D – represented by open boxes (see also [18]). In addition, the origin of the 3.0 kb *Eco*RI fragment forming the recombinant plasmid pLG579 [7] is shown. The construction of pLG609 is described in section 2.3. *Abbreviations:* B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; Ps, *Pst*I; Pv, *Pvu*II.

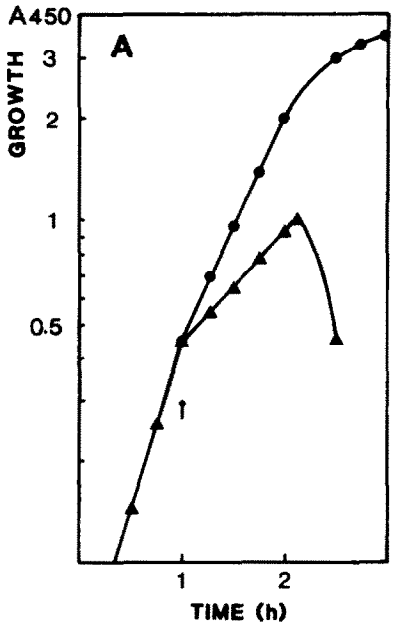
(pLG609) were studied in the presence or absence of pLG575 [7], a plasmid carrying *hly*B and *hly*D, and with the addition of IPTG in order to induce synthesis of Ac from the *tac* promoter. Fig.2A shows that addition of IPTG to a strain carrying pLG609 with no *hly* export functions resulted in rapid cessation of growth. In contrast, when the export functions were also present the addition of IPTG had little effect on growth. This result suggested that the intracellular accumulation of a polypeptide, most probably Ac, in the absence of Hly export proteins was toxic for the cell and that this effect might be alleviated by export of the Ac protein to the medium (see section 3.3).

3.3. *Secretion of a 23 kDa protein encoded by pLG609*

The protein content of the culture medium from the experiment shown in fig.2A was analysed by SDS-PAGE. Only small amounts of a 23 kDa polypeptide were induced in the total cell lysate of strain SE5000 carrying only pLG609 and no 23 kDa protein was detected in the culture supernatant (not shown). In contrast, however, fig.2B shows that in the presence of the export functions substantial quantities of a 23 kDa polypeptide accumulated in the medium after addition of IPTG.

3.4. *Comparison between the efficiency of secretion of the 107 kDa HlyA protein and the 23 kDa Ac polypeptide*

We have previously shown that during exponential growth haemolysin secretion from *E. coli* K12 strains is extremely efficient [7] and that the vast majority of the 107 kDa, HlyA protein is recoverable from the culture supernatant [13]. Thus, in order to compare the amount of the 107 kDa protein secreted with that of the 23 kDa, Ac polypeptide, whole cell and culture supernatant samples were prepared as described in section 2.4. Fig.3 shows the results of analysis of these samples by SDS-PAGE and the subsequent identification of the proteins using antiserum to purified haemolysin [17]. Clearly, the ratio of intracellular



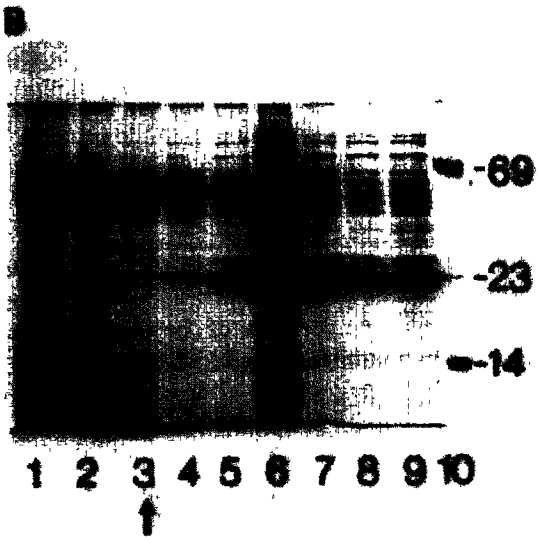


Fig.2. (A) Growth of *E. coli* SE5000 (pLG609) following the addition of IPTG at time 1 h to derepress the *tac* promoter; (●) *E. coli* (pLG609) also carrying the plasmid pLG575 which encodes intact *hly*B and *hly*D necessary for export of haemolysin; (▲) *E. coli* (pLG609) without any export functions. (B) Culture supernatant samples were prepared from the growth cycle of *E. coli* SE5000 (pLG609/pLG575) shown in A. Each track represents analysis of proteins present in 10 ml of supernatant by SDS-PAGE (15% acrylamide) followed by staining with Coomassie brilliant blue. The position of the 23 kDa, C-terminal fragment of HlyA (Ac) is indicated and is clearly induced by the addition of IPTG.

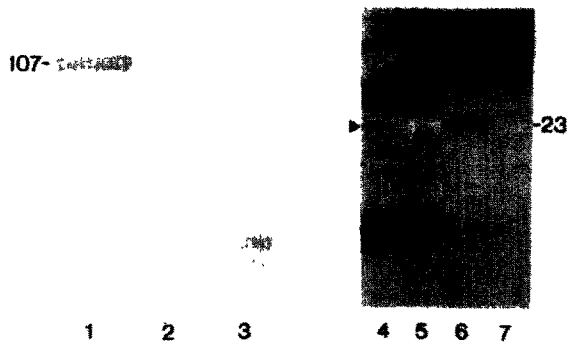


Fig.3. This figure shows the efficiency of secretion of the 107 kDa, HlyA protein compared with that of the 23 kDa, Ac peptide. As far as possible, proteins from either the culture supernatant or whole cells were prepared from the same volume of cells so that the amount of any particular protein was directly comparable on the Western blot. In all lanes, except track 1 where 10 times the amount was loaded, protein samples were prepared from 1 ml cells harvested at A_{450} = 1.0. Lanes: 1, supernatant (SN) SE5000 (pLG570); 2, whole cells (WC) SE5000 (pLG570); 3, WC SE5000; 4, WC SE5000 (pLG609); 5, WC SE5000; 6, SN SE5000 (pLG609); 7, SN SE5000.

to extracellular protein indicates that once induced, the 23 kDa polypeptide is apparently secreted with an efficiency similar to that of the 107 kDa polypeptide.

4. DISCUSSION

The results presented here demonstrated unequivocally that the C-terminal, 23 kDa peptide of HlyA contains all the information necessary for its own secretion. As expected, secretion was only observed in the presence of the specific haemolysin export proteins, HlyB and HlyD, and does not require HlyC (see also [8]). These results strongly suggest that a specific region close to the C-terminus contains all the information necessary for secretion of the intact *E. coli* haemolysin. Our results are therefore consistent with a mechanism in which the C-terminal region of HlyA specifically interacts with HlyB and HlyD in order to facilitate secretion. Previous studies indicated that at least the HlyD protein is located primarily within the inner membrane [18] and consequently, we have proposed a model in which the toxin is secreted direct-

ly to the medium from the cytoplasm either through proteinaceous pores formed between inner and outer membranes or via a vesicular intermediate [13].

The finding that the secretion signal(s) of HlyA is (are) probably located in a small C-terminal domain should now greatly simplify attempts to understand the nature of the secretion process by showing specific interactions between the toxin and export proteins. In addition, other studies will be directed towards the identification of the smallest fragment from the C-terminus which carries information sufficient for its own secretion. The presence of a topogenic sequence so close to the C-terminus is quite unique. Moreover, inspection of the sequence data [11] does not reveal any conventional form of signal sequence. In fact, in contrast to the export of most proteins across membranes which has been shown to involve N-terminal signals, we have found that secretion of haemolysin is unaffected by *sec*A (unpublished).

We suggest that the apparently highly specific nature of haemolysin transport rests primarily upon the initial recognition of the export machinery by the C-terminal region of the HlyA protein. Nevertheless, despite its apparently novel features this well defined system provides an excellent opportunity to establish the precise details of the actual membrane translocation event – a mechanism which remains elusive in other systems – with the expectation that some more broadly applicable principles will emerge.

ACKNOWLEDGEMENTS

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