Characterisation of HlyC and mechanism of activation and secretion of haemolysin from *E. coli* 2001

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In this paper the DNA sequence of the cloned *hlyC* gene from *E. coli* 2001 is presented. The gene encodes a protein of 20 kDa which is able to activate the 107 kDa polypeptide encoded by *hlyA*. This gives rise to a haemolytically active protein which differs from the inactive form in stability and by its migration when analysed by polyacrylamide gel electrophoresis under non-denaturing conditions. We also show that the inactive form is secreted in the presence of the transport functions *hlyB* and *hlyD*. This result rules out any role for the *hlyC* gene product in the transport of HlyA across the inner membrane

Hemolysin secretion *Post-translational modification* hlyC *sequence*

1. INTRODUCTION

The haemolysin determinant is composed of an approx. 7 kb region encoding 4 genes. A 3 kb region encoding *hlyC* and *hlyA* determines the synthesis of a haemolytically active protein whilst a contiguous 4 kb region encodes *hlyB* and *hlyD* (nomenclature adopted from [1], corresponding to $hlyB_a$ and $hlyB_b$, respectively) which are essential for export of the haemolysin to the culture medium [2–4].

Goebel and co-workers [5] proposed that the *hlyC* gene is involved in a proteolytic cleavage of the 107 kDa polypeptide encoded by *hlyA* and the concomitant transport of a haemolytically active 58 kDa derivative across the inner membrane. From the periplasm, it was proposed that this protein is then transported across the outer membrane by the export functions encoded by *hlyB* and *hlyD*.

In contrast, we have shown that the extracellular form of the haemolysin is a 107 kDa polypeptide [3,6]. In addition we now show for the first time that some form of post-translational modification of haemolysin is effected by *hlyC* which leads to the activation of the 107 kDa haemolysin. We also show that this protein is transported to the medium in the complete absence of HlyC.

2. MATERIALS AND METHODS

2.1. *Strains*

The laboratory *Escherichia coli* K12 strain MC4100 *rpsL*, *ara*139, Δ(*lac*IPOZA) *u*169, *thi* was used in all of these studies. The plasmid pLG570 contains the haemolytic determinant from LE2001 isolated as described in [3]. The plasmids pLG575 and pLG583 have been described elsewhere (Mackman et al., submitted). Plasmid pUC12 has been described by Messing [7].

2.2. *Media*

Cultures were grown in rich medium (Luria broth) in the presence of 10 mM calcium chloride.

2.3. *Gene expression system*

The in vitro transcription-translation procedure using plasmid DNA templates was carried out as in [8].

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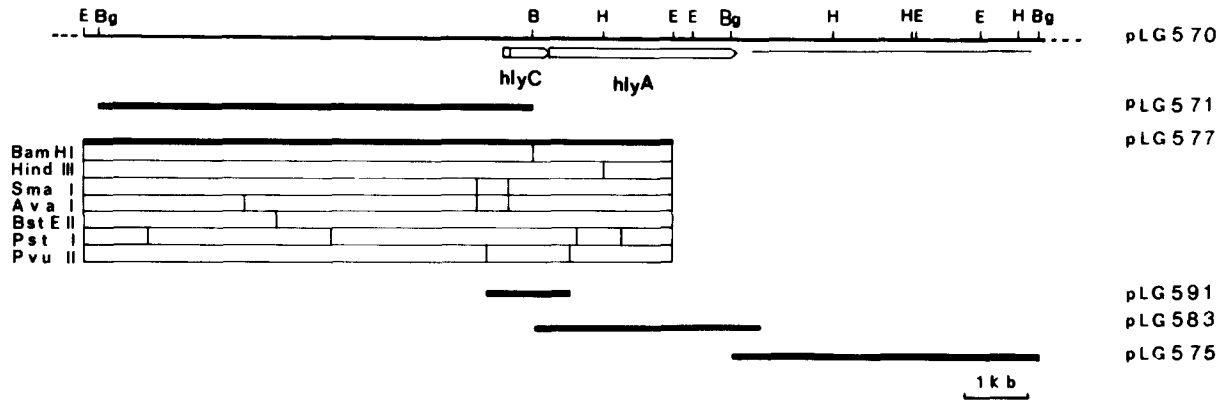

Fig.1. Restriction endonuclease map and genetic organization of the Hly determinant LE2001. pLG570 containing the *hly* determinant from a human isolate *E. coli* LE2001 is shown with the *hlyC* and *hlyA* cistrons placed according to transposon mapping and complementation analysis (Mackman et al., submitted). pLG571 and pLG577 are also shown. A restriction endonuclease map of pLG577 was obtained by partial digestion of end-labelled DNA as described [14]. The 1.4 kb *Pvu*II fragment containing the *hlyC* gene was cloned into the *Sma*I site of pUC12 to form the recombinant plasmid pLG591. The construction of plasmid pLG575 containing the export functions and plasmid pLG583 containing *hlyA* is described elsewhere (Mackman et al., submitted). (E) *Eco*RI, (B) *Bam*HI, (Bg) *Bgl*II, (H) *Hin*dIII.

2.4. *DNA sequencing*

This was performed according to the Sanger technique using the M13 mp8/mp9/mp18 system [9] as described in [10].

2.5. *Stability of the 107 kDa protein*

The stability of [^{35}S]methionine-labelled 107 kDa protein was measured by spectroscopic scanning of autoradiograms as in [11].

3. RESULTS

3.1. *Cloning of* hlyC *and identification of its gene product*

A restriction map of the plasmid pLG570 containing *hly* determinant from the human haemolytic strain LE2001 [3] is shown in fig.1. Fig.1 also shows further detailed restriction site analysis of the region surrounding *hlyC*. A previous complementation analysis localised 2 genes *hlyA* and *hlyC* required for intracellular haemolytic activity in pLG570. To characterise the *hlyC* gene, the *Eco*RI and the *Bgl*II-*Bam*HI

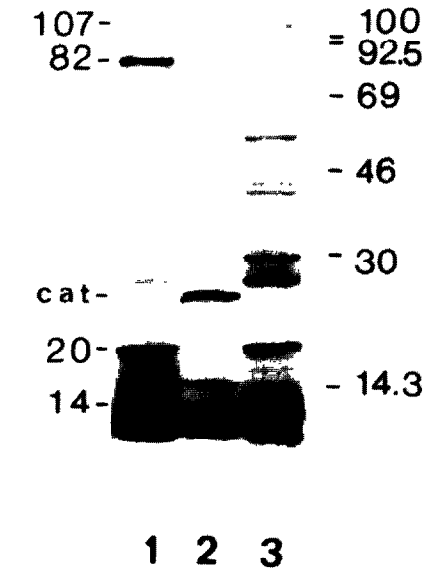

Fig.2. Plasmid DNA of pLG570, and the subclones pLG571 and pLG577 were used to programme an in vitro coupled transcription-translation system and proteins labelled with [^{35}S]methionine. These proteins were visualised by autoradiography after SDS-PAGE (11% acrylamide). Track 1, pLG577; track 2, pLG571; track 3, pLG570. Note the 14 kDa polypeptide in track 2 is not well resolved using an 11% gel. Molecular masses are given in kDa.

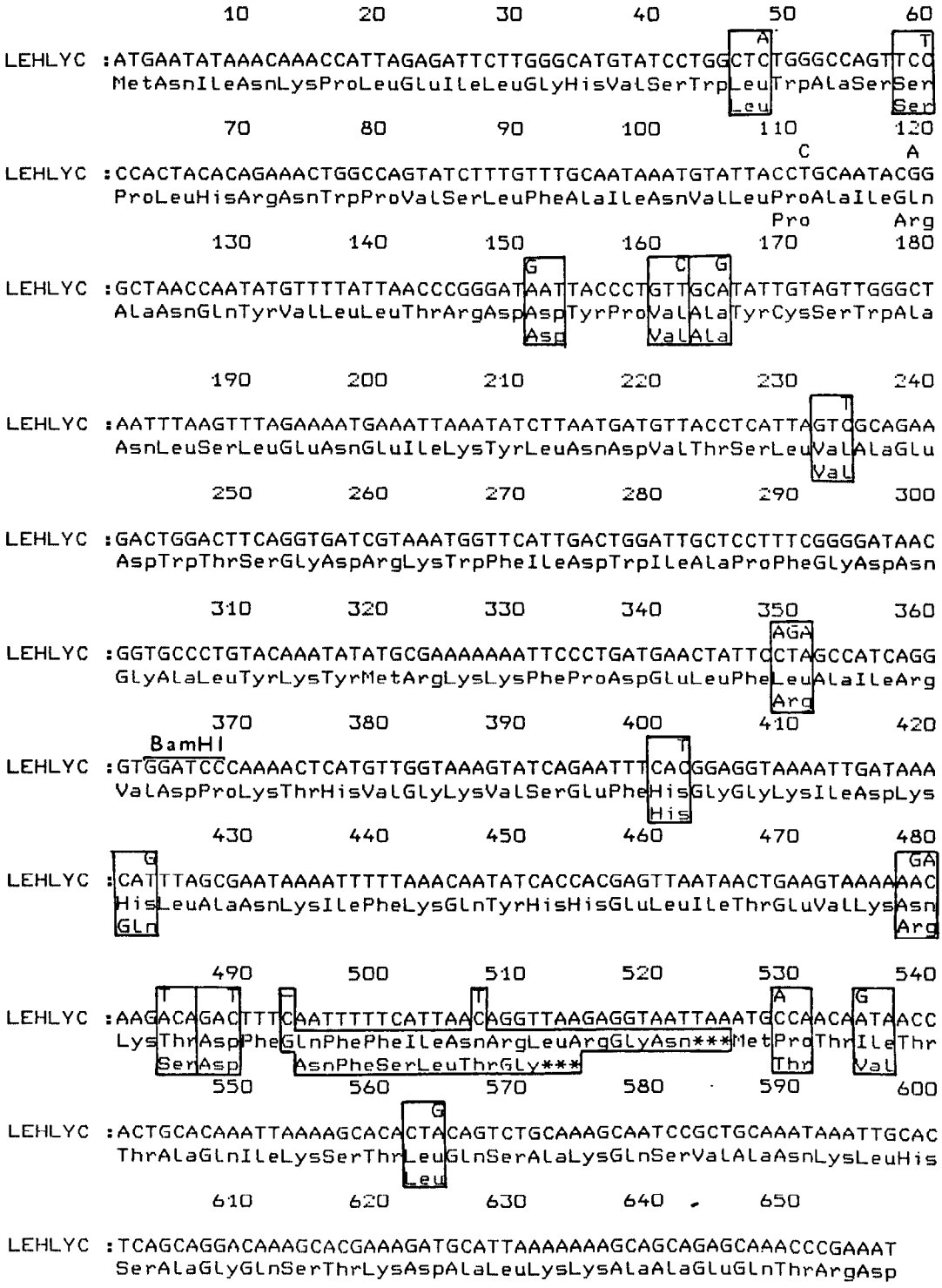

Fig.3. Nucleotide and amino acid sequences of the *hlyC* gene and the 5′-end of the *hlyA* gene of the chromosomal haemolytic determinant LE2001. The sequences were compared with the sequences of the *hlyC* gene [12] and the 5′-end of the *hlyA* gene [5] of the haemolytic determinant of animal origin coded by the plasmid pHly152. The position of the *Bam*HI site shown in fig.1 is indicated.

fragments of pLG570 were cloned into the *Eco*RI and the *Bam*HI sites of pACYC184 to form the recombinant plasmids pLG577 and pLG571, respectively (fig.1). Plasmid pLG577 complements the *hlyA* (pLG583) and the export functions *hlyB, hlyD* (pLG575) in vivo to produce haemolytic clones whilst pLG571 fails to produce haemolytic clones under these conditions. The analysis of the proteins synthesised in vitro with these recombinant plasmids is shown in fig.2. Plasmid pLG577, directed the synthesis of a 20 kDa protein corresponding to the *hlyC* gene product. In addition, this fragment directed the synthesis of an 82 kDa polypeptide which appears to be a protein fusion containing part of the Cat protein and the N-terminal region of the 107 kDa polypeptide. In contrast, pLG571 failed to direct the synthesis of a 20 kDa protein but a new 14 kDa protein was detected which appears to be a truncated form of *HlyC*, resulting from cloning the *Bgl*II-*Bam*HI fragment. In consequence a 1.4 kb *Pvu*II fragment containing the *Bam*HI site identified above was inserted into the *Sma*I site of pUC12. The resulting recombinant plasmid pLG591, cotransformed with pLG583, which encodes *hlyA*, resulted in the production of internal haemolytic activity demonstrating the presence of a functional *hlyC* gene present in the *Pvu*II fragment.

3.2. *Sequence of the* hlyC *gene*

The relevant portion of the 1.4 kb *Pvu*II fragment surrounding the *Bam*HI site was sequenced by the Sanger technique. Few differences were found from the published *hlyC* sequence [12] and therefore only a low percentage of the DNA was sequenced from both strands. Fig.3 shows the DNA sequence and the predicted amino acid content encoded by *hlyC*. This is also compared with the *hlyC* gene encoded by the haemolytic plasmid pHly152 of animal origin [12]. The two genes are remarkably homologous with 97% of the DNA sequence being conserved and 92% of the amino acid sequence similarly conserved. The most important difference in the DNA sequence from Hly 2001 results in the presence of an additional C nucleotide at position 493 which gives a different reading frame for the C-terminal region compared with the HlyC protein of pHly152. This results in the HlyA protein translation initiation codon being immediately after the stop codon of the *hlyC* gene. Inspection of the amino acid sequence of HlyC indicates the presence of a hydrophobic domain between residues 27 and 48. However, preliminary localisation studies suggest that the polypeptide is not an integral membrane protein and the polypeptide is identical in size when synthesised in vitro and in vivo demonstrating that proteolytic processing of the N-terminal region does not take place.

3.3. *The role of* hlyC *in the production of active haemolysin*

We have constructed subclones in which *hlyA* is fully expressed (as determined by the use of HlyA antiserum) from the *tet* promoter in the absence of *hlyC* (Mackman et al., submitted). This indicates that HlyC is not required at the translational level for the expression of *hlyA*.

To test the role of HlyC in the export of HlyA a strain was constructed carrying the plasmid

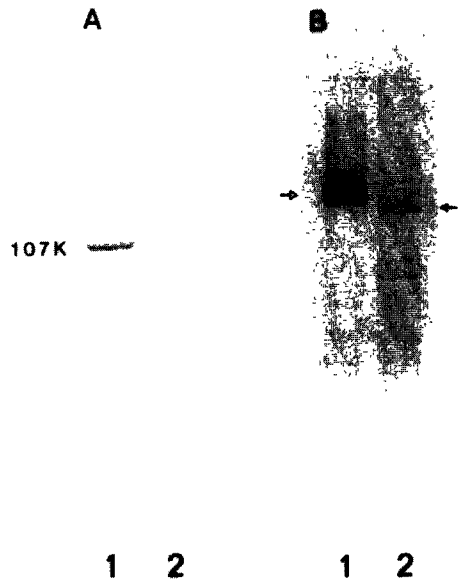

Fig.4. Comparison of the modified and the unmodified form of the haemolysin. Supernatant samples from late exponentially growing cultures were analysed by SDS-PAGE (11% acrylamide) in A or under non-denaturing gel electrophoresis conditions (11% acrylamide) in the absence of SDS, B. Track 1, non-haemolytic, unmodified form (—▷) of HlyA produced by MC4100, pLG575 + pLG583; track 2, the modified form (◁—) produced by MC4100, pLG570.

pLG583 (*hlyA*), together with pLG575 which encodes all the export functions. Although such clones are completely non-haemolytic substantial amounts of a 107 kDa protein were found in the medium (fig.4). The identity of this protein as the haemolysin was confirmed by precipitation with antibody (not shown). Therefore, although the HlyC protein is required for activation of the HlyA protein it is not required for its secretion to the medium. When the two protein products, active and inactive 107 kDa protein, were compared by SDS-PAGE (fig.4A) no differences in size were detected. However, as shown in fig.4B when the two forms were analysed in non-denaturing, 11% acrylamide gels lacking SDS, the active form clearly had a greater relative mobility, indicating an increased negative charge. Moreover, the inactive, 107 kDa protein was relatively stable (fig.5), with a half-life of 42 min, when pulse-chase experiments were carried out with MC4100 (pLG575 + pLG583). We have shown previously that the half-life of active haemolysin is maximally 25 min under these conditions [11].

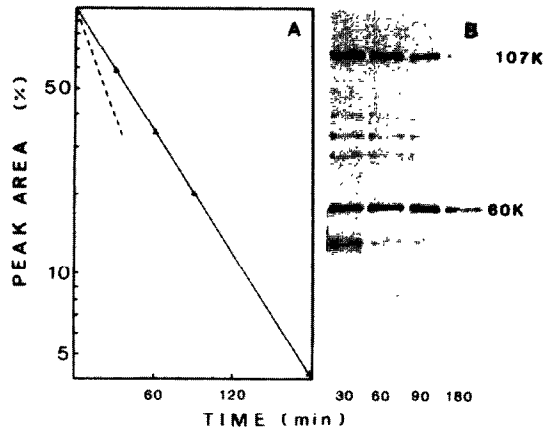

Fig.5. Stability of the unmodified 107 kDa polypeptide in the culture medium. An exponentially growing culture of strain MC4100 (pLG583 + pLG575), grown in M9-glucose minimal medium at 37°C to A_{600} = 1.0, was pulse-labelled with [^{35}S]methionine (50 μCi for 5 min) and chased with an excess of cold methionine in the presence of 250 μg/ml chloramphenicol. The 107 kDa polypeptide (uppermost band) was precipitated at various times, and analysed by SDS-PAGE (11% acrylamide) followed by autoradiography (B). In A the amount of radioactivity in the 107 kDa band, determined by scanning the gel in B with an LKB Ultrascan (model 2202), is presented. Stability of the modified 107 kDa polypeptide is presented as a broken line.

4. DISCUSSION

The *hlyC* gene, together with the structural gene for haemolysin, *hlyA* are both required for the production of active haemolysin. However, we now show that the 20 kDa product of *hlyC* is not required for either the synthesis or the export of the 107 kDa, *hlyA* gene product, but is responsible for modification of the 107 kDa polypeptide which renders it haemolytic. This activation increases the mobility of the 107 kDa protein in non-denaturing gels and increases its sensitivity to proteolytic degradation. Preliminary results indicate that the modification of the 107 kDa polypeptide does not involve either glycosylation or phosphorylation of the protein.

Previous findings that the haemolysin secreted by *E. coli* is the 107 kDa primary product of the *hlyA* gene [3,6] together with the present data that HlyC is not required for export of this polypeptide are quite inconsistent with previous models of haemolysin secretion [5]. Further studies, particularly in regard to the nature and function of the *hlyB* and *hlyD* export functions are now essential to further understanding of the precise export mechanism.

Comparison of the sequence of the *hlyC* gene of the human haemolytic determinant LE2001 with the *hlyC* gene encoded by the plasmid pHly152 of animal origin revealed 97% homology. These data confirmed previous indications that *E. coli* haemolysin determinants are strongly conserved [13] and that they have probably evolved from a common determinant.

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