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Analysis of the haemolysin secretion system by PhoA-HlyA fusion proteins*

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Summary. We studied the efficiency of the pHly152-derived haemolysin transport system using PhoA-HlyA fusion proteins and different constructs which provide HlyB/HlyD *in trans*. The optimal C-terminal HlyA signal consists of the last 60 amino acids. Longer stretches of HlyA do not improve the transport efficiency of PhoA-HlyA fusion proteins. The introduction of deletions and/or replacements in the 60 amino acid HlyA signal domain revealed at least three functional regions with different degrees of specificity. Amino acids 1–21 (numbered from the N-terminal part of the 60 amino acid HlyA signal), termed region I, could be replaced by a Pro-containing peptide. The other two regions II and III (amino acids 22–40 and 41–60, respectively) seem to interact directly with the HlyB/HlyD translocator since a PhoA fusion protein which contains either of the two regions was still secreted in a HlyB/HlyD-dependent mode, albeit at low efficiency. An efficient *trans*-complementing HlyB/HlyD system was only obtained from the pHLy152-encoded *hly* determinant when the regulatory *hly*R element was provided *in cis*. Secretion of the PhoA-HlyA fusion protein did not interfere with the secretion of HlyA even when the fusion protein was induced to a high level. This suggests that the capacity of the HlyB/HlyD translocation system is high and not normally saturated by its natural HlyA substrate.

Key words: *Escherichia coli* haemolysin – Secretion – HlyA signal sequence – Fusion proteins

Introduction

Transport of proteins across bacterial cytoplasmic membranes occurs by a mechanism similar to protein translocation across the endoplasmic reticulum in eukaryotes. In both cases an N-terminal signal peptide allows the precursor protein molecule to recognize the transport machinery. The N-terminal signal peptides, although highly variable in their primary amino acid sequences, contain common characteristic features (Lee and Beckwith 1986; Pugsley and Schwartz 1985; Randall et al. 1987) which are similar in pro- and eukaryotic signal peptides. During translocation of the polypeptide chain across the membrane the N-terminal signal sequence is cleaved off the precursor molecule by signal proteases, which belong to the membrane-associated portion of the protein export machinery (Lee and Beckwith 1986). The other part of this machinery is cytoplasmic and includes, in the case of eukaryotes, the well characterized signal-recognition particle (SRP). The components of the protein transport machinery have been extensively characterized in eukaryotes, mainly in mammalian cells (Walter and Lingappa 1986; Saier et al. 1989). In prokaryotes the components of the export machinery are less well known but the isolation of conditional mutants defective in protein transport (sec-mutants) is compatible with a similar composition of the protein translocation complex (Wolfe et al. 1985; Oliver and Beckwith 1981).

In contrast to protein transport across the inner membrane, the secretion of proteins into the medium by gram-negative bacteria i.e. the translocation of a protein across two membranes, proceeds by different mechanisms (Pugsley and Schwartz 1985; Holland et al. 1989). The only two types of *Escherichia coli* proteins known to be translocated through the inner and the outer membranes are the colicins and α-haemolysin. Whereas colicins may not use a specific transport machinery (Pugsley and Schwartz 1985; Baty et al. 1987), transport of α-haemolysin is accomplished by a specific system consisting of the two membrane proteins HlyB and HlyD (Wagner et al. 1983). In addition, the transported protein HlyA, a rather hydrophilic protein of 110 kDa (Mackman et al. 1984; Goebel and Hedgpeth 1982), carries a specific recognition sequence for this translocation system that is located in the C-terminal region of HlyA. This was first demonstrated by deletions introduced into the C-terminal part of HlyA (Härtlein et al. 1983; Nicaud et al. 1986; Koronakis et al. 1989). More recently,

* Dedicated to Prof., Dr. F. Lingens on the occasion of his 65th birthday

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fusion proteins were constructed which consisted of different reporter proteins (Holland et al. 1989; Mackman et al. 1987; Gentschev et al. 1990; Hess 1990) to which various regions of the C-terminal part of HlyA were linked. Translocation of these fusion proteins by HlyB and HlyD could be accomplished, albeit often at reduced efficiencies.

In addition to these authentic *E. coli* secretory proteins, secretion in *E. coli* has also been obtained with other heterologous proteins. The genes coding for these proteins were derived from other gram-negative bacteria and were introduced into *E. coli*. The IgA protease of *Neisseria gnorrhoeae* (Pohlner et al. 1987) and the neutral protease of *Serratia marcescens* (Yanagida et al. 1986) carry, in addition to a conventional N-terminal signal peptide, a C-terminal "helper sequence" which ensures its translocation across the outer membrane. Pullulanase of *Klebsiella pneumoniae* (Pugsley and Schwartz 1985; Pugsley and Reyss 1990) is synthesized as a precursor polypeptide with an N-terminal signal peptide which is processed by lipoprotein signal peptidase during its translocation across the cytoplasmic membrane. This suggests that pullulanase, like IgA protease, is translocated across the cytoplasmic membrane of *E. coli* via the general export pathway (sec-pathway). The sec-pathway is, however, insufficient to translocate pullulanase across the outer membrane. Recent data (Pugsley and Reyss 1990) suggest that at least five specific *Klebsiella* genes are required for this step. Although these foreign proteins are thus secreted by different mechanisms, they all employ the sec pathway for the first step, i.e. the translocation across the inner membrane. *E. coli* haemolysin appears to be secreted independently of the sec pathway (Holland et al. 1989; Gentschev et al. 1990) and translocation of haemolysin seems to occur simultaneously across both membranes. This suggests that HlyB/HlyD may form a membrane channel through which haemolysin passes without entering the periplasmic space. The previous identification of periplasmic pools of haemolysin in *hly*B$^-$ and *hly*D$^-$ mutants (Härtlein et al. 1983) can be explained by the membrane-active amphiphilic N-terminal end of HlyA (Erb et al. 1987) which may allow some leakage of haemolysin through the inner membrane under certain conditions. Recent data (Oropeza-Wekerle et al. 1990) suggest that HlyB alone can channel HlyA into the two membranes of *E. coli*. Under these conditions most of the HlyA molecule remains, however, in the membrane and only a small part, probably the C-terminal end of HlyA, is exposed on the surface. This observation suggests that HlyB recognizes the C-terminal signal sequence of HlyA and directs the HlyA polypeptide into the membrane.

In order to study further the interaction between the HlyB/HlyD translocation system and the C-terminal recognition signal of HlyA, we have constructed a PhoA fusion protein that contains the minimal C-terminal peptide providing optimal transport efficiency in the presence of HlyB/HlyD. The analysis of this system indicates three functional regions in the 60 amino acid signal sequence of HlyA which are differentially sensitive to alterations in their amino acid sequence. The development of a *trans*-complementing plasmid efficiently expressing HlyB and HlyD, and derived from the plasmid-encoded *hly* determinant, required the promoter region of the *hly* determinant and the *cis* activator element *hlyR*. It is further shown that the capacity of the HlyB/HlyD translocation system is high and it is able to secrete large amounts of fusion proteins without affecting the efficiency of transport of its natural substrate, HlyA.

Materials and methods

Strains. The bacterial strains used were *E. coli* CC118 *ara*D139, (*ara, leu*) 7697 *Δlac* X74 *phoA* Δ20 *gal*E *galK thi rpsE rpoB argE*am *recA1* (Manoil and Beckwith 1985) and *E. coli* HB101 *hsdS20* (r_b^- m_b^-) *ara14 proA2 lacY1 galK2 rpsL20 xyl15 mtl1 supE44 recA13* (Boyer and Roulland-Dussoix 1969). Bacterial strains were grown with aeration at 37° C in 2× YT. For plasmid-containing strains, chloramphenicol (30 μg/ml) or ampicillin (50 μg/ml) was added as appropriate.

Enzymes and chemicals. The restriction endonucleases and DNA modifying enzymes were purchased from Boehringer (Mannheim, FRG) or Pharmacia (Uppsala, Sweden) and used as recommended. All other chemicals were supplied by Merck (Darmstadt, FRG), Roth (Karlsruhe, FRG), Serva (Heidelberg, FRG) and Sigma (Deisenhofen, FRG). ^{32}P-ATP was acquired from Amersham (Braunschweig, FRG). Powdered growth media were obtained from Difco (Detroit, USA).

Computer software. The programs for DNA and protein analysis were purchased from Genofit (Geneva, Switzerland).

Oligonucleotides. Oligonucleotides for cloning and sequencing were synthesized using a DNA synthesizer from Applied Biosystems (Weiterstadt, FRG). The oligonucleotides *M28* (GCCGGGTGCAGTAATATC) and *M29* (CTTATGTGGCGTCGACAGCCCAG) were used for sequencing and the oligonucleotides *M44* (CCCGGGTTACAAACTGCAATAAAGAAGCTG-CA) and *M49* (CTGCAGTTCTTTCCTCTTTAA-CATCGAAGCTACCTGCATG) were template strands in a specific cloning method described previously (Carter-Muenchau and Wolf 1987).

Plasmids. The haemolysin export genes *hlyB* and *hlyD* are encoded on the plasmids pBD152 and pBD1c. Details of the construction of the recombinant plasmids are given in the text and Fig. 1. The plasmid pAL-C2 is a derivative of pANN202-312AL3* described previously (Ludwig et al. 1987). A *Cla*I deletion has altered the DNA sequence upstream of the *hlyR* element (Vogel et al. 1988). Detailed descriptions of the plasmids pJHH-700B, pJHH-600B, pPhoA, pJHH-575B, pJHH-537, pJhh-500B, pJHH-500BL, pJHH-350B and pJHH-300B are given in the text and Figs. 3 and 6B. Specific deletions in some plasmids derived from pJHH-600B were obtained by successive cloning of appropriate oligonucleotides according to Carter-Muenchau and Wolf (1987).

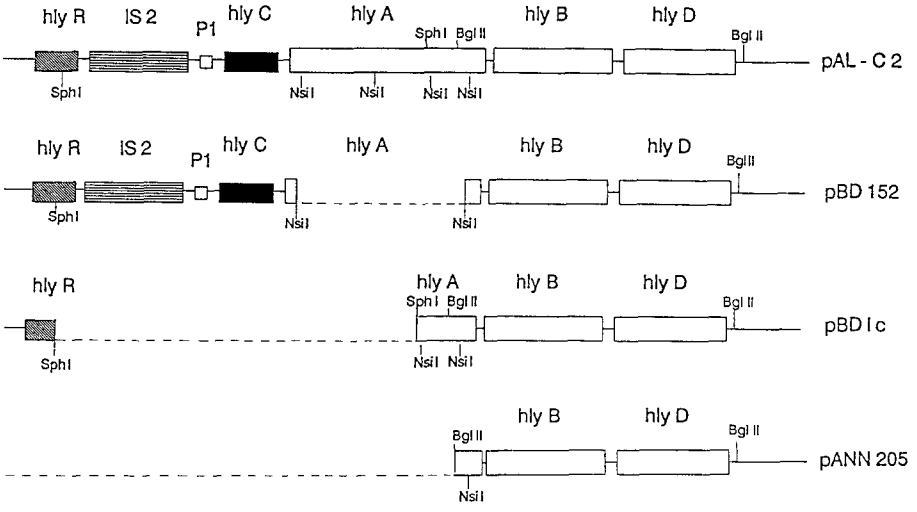

Fig. 1. Recombinant plasmids carrying the genes *hlyB* and *hlyD* necessary for secretion of haemolysin. Plasmid pAL-C2 is essentially identical to pANN202-312AL3* (Ludwig et al. 1987) with pACYC184 as vector; pBD152 and pBD1c are derivatives of pAL-C2 generated by deletion of internal *Nsi*I and *Sph*I fragments, respectively. pANN205 carries the *Bgl*II fragment of pANN202-312 (Hess et al. 1986) cloned into the *Bam*HI site of pACY184

Preparation of bacterial cell extracts and culture supernatant samples. Bacterial cultures were grown in 2× YT medium overnight at 37° C and diluted 20-fold into fresh medium in the presence of the appropriate antibiotic and grown to early log phase ($A_{600}=0.4$) before IPTG (Sigma) was added to a final concentration of 1 mM. Cell samples were prepared by harvesting the cells and resuspending them directly in SDS sample buffer (Laemmli 1970). Culture supernatant samples were prepared by first removing the cells by centrifugation (Beckman JA20, 15000 rpm, 15 min) and then adding trichloroacetic acid (TCA) to a final concentration of 7% (w/v) to the supernatant. Precipitated proteins were harvested by centrifugation (Beckman JA20, 8000 rpm, 20 min) and the TCA was neutralised using a saturated TRIS solution before proteins were solubilised in SDS sample buffer.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting procedure. Protein samples were fractionated by SDS-PAGE on 10% or 12.5% polyacrylamide gels (Laemmli 1970). Gels were stained with Coomassie Brilliant Blue R250 or silver nitrate (Oakley et al. 1980). Proteins were transferred to nitrocellulose filters as described previously (Towbin et al. 1979) and incubated with anti-alkaline phosphatase antibody. Proteins reacting with the polyclonal rabbit antibodies were visualized after the horseradish peroxidase reaction using the substrate 4-chloro-1-naphthol.

DNA sequencing. Altered DNA sequences of the various plasmid constructions were determined with the T7 polymerase sequencing kit from Boehringer (Mannheim, FRG) using appropriate oligonucleotides.

Results

Construction of an efficient trans*-complementing HlyB/HlyD system derived from pHly152*

As previously shown (Vogel et al. 1988) efficient transport of haemolysin (HlyA) by the pHly152-encoded haemolysin (*hly*) determinant is only achieved when the *cis*-acting activator sequence *hlyR* is present together with the promoter region upstream of *hlyC*. The activating function of *hlyR* mainly affects transcription of the transport-mediating functions *hlyB* and *hlyD*. This effect seems to be caused by antitermination of transcription at a terminator sequence beyond *hlyA* (Koronakis et al. 1988). Plasmid pANN202-812/17, which carries *hlyR*, *hlyC* and *hlyA* (Oropeza-Wekerle et al. 1989), expresses a large amount of haemolysin which is not secreted. We have used this plasmid to develop construct containing *hly*B and *hly*D which can provide efficient transport functions for haemolysin encoded by pANN202-817/17 *in trans* (Fig. 1). Plasmid pANN205 carries the *hly* sequence from nucleotides 3811–10490, which includes 584 bp of *hlyA* (Hess et al. 1986), the terminator sequence and the two transport genes *hlyB* and *hlyD*. This *Bgl*II fragment was cloned into the *Bam*HI site of pBR322 and pACYC184 such that expression of *hlyB* and *hlyD* is under the control of the *tet* promoter. As shown in Fig. 2, *HlyA* is not exported to an appreciable extent from *E. coli* HB101 strains which contain pANN202-812/17 together with either of these two constructs. Plasmid pBD152 (Fig. 1) was derived from pAL-C2 by deleting most of *hlyA*. This plasmid contains the intact transcriptional control region of the *hly* determinant, *hlyC*, *hlyB* and *hlyD*, with the same intergenic regions as the original plasmid pAL-C2. Figure 2 shows that this plasmid provides efficient transport functions *in trans* in *E. coli* HB101 carrying pANN202-812/17. The derivative pBD1c contains an essential portion of *hlyR* (Vogel et al. 1988), a small region of the 3′ end

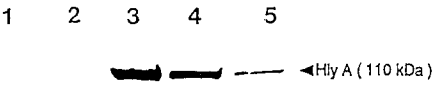

Fig. 2. External haemolysin (HlyA protein, 110 kDa) after SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining secreted by *Escherichia coli* 5K carrying pANN202-812/17 (Oropeza-Wekerle et al. 1989) alone (lane 1) and this plasmid complemented with pANN205 (lane 2), pAL-C2 as control (lane 3), pBD152 (lane 4) and pBD1c (lane 5)

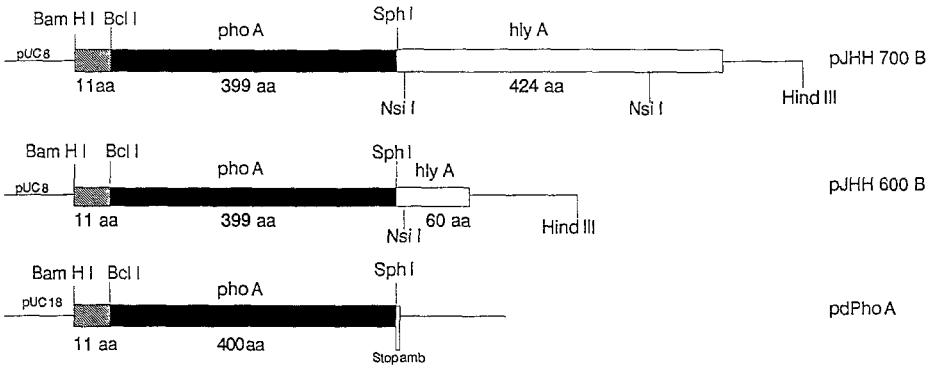

Fig. 3. Recombinant plasmids expressing PhoA-HlyA proteins with the C-terminal 424 amino acids (pJHH700B) and 60 amino acids (pJHH600B) of HlyA. Plasmid pdPhoA expresses a deletion derivative identical at the N-terminal end to the latter PhoA-HlyA proteins but lacks a C-terminal HlyA sequence. All three constructs lack the original N-terminal leader sequence of PhoA and carry instead 11 amino acids from the polylinker sequence of the vector

of *hlyA*, the transcription terminator beyond *hlyA* and the two transport genes *hlyB* and *hlyD*. This plasmid allows transport of HlyA expressed from pANN202-812/17 when provided *in trans*. The transport efficiency obtained with pBD1c in this experiment is, however, lower than that obtained with pBD152. For the secretion studies with the PhoA-HlyA fusion proteins described in the following we have therefore used pBD152 or pAL-C2. The latter plasmid expresses full-length HlyA and complementation of a recombinant plasmid expressing PhoA-HlyA with pAL-C2 therefore allows comparison of the transport efficiency of the fusion protein with that of the HlyA protein.

Construction of PhoA-HlyA fusion proteins and analysis of their transport efficiency mediated by HlyB/HlyD

Using the strategy outlined in Fig. 3 the two plasmids pJHH700B and pJHH600B were constructed. These plasmids encode PhoA-HlyA fusion proteins which are identical at their N-terminal ends but carry, at their C-terminal ends, 424 and 60 amino acids, respectively, derived from the C-terminal part of HlyA. In addition, plasmid pPhoA was constructed; it lacks the HlyA portion but encodes a PhoA derivative with the same N-terminal end as those encoded by pJHH700B and pJHH600B. All three PhoA derivatives have lost 49 amino acids at the N-terminus, including the PhoA-specific signal sequence, which is replaced by 11 amino acids unrelated to PhoA, as outlined in Fig. 3. At the C-terminal end the last 35 amino acids of PhoA are replaced by the HlyA-derived amino acids in the case of PhoA-HlyA700B and PhoA-HlyA600B. In the case of protein PhoA-del, used as a control, the C-terminal 35 amino acids are missing. Expression of all three PhoA derivatives is under the control of p*lac* and synthesis of these proteins can therefore be induced by IPTG. As expected, the control protein PhoA-del was not transported at all. In the presence and absence of pAL-C2 similar amounts of internal PhoA-del protein can be detected with anti-PhoA antibodies but no secretion of this protein occurs under these conditions (Fig. 4). No transport of PhoA-HlyA600B or PhoA-HlyA700B is observed in the absence of HlyB/HlyD, however similar amount of each

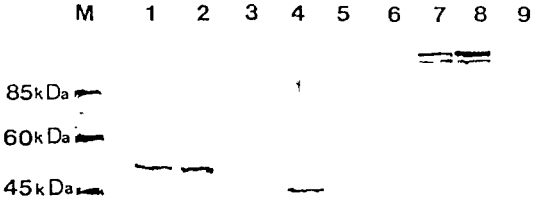

Fig. 4. Immunoblots showing the internal and external PhoA-HlyA fusion proteins expressed by pJHH600 (lanes 1–3), pdPhoA (lanes 4–6) and pJHH700B (lanes 7–9). Transport functions were provided by pAL-C2 (in lanes 1, 2, 4, 5, 7, 8) or were missing (lanes 3, 6, 9). Lanes 1, 4 and 7 show internal, lanes 2, 3, 5, 6, 8 and 9 external PhoA-derivatives. PhoA-derivative proteins were visualized with polyclonal anti-PhoA antibodies as described (Gentschev et al. 1990). The amount of protein in each lane was normalized to the same number of bacterial cells

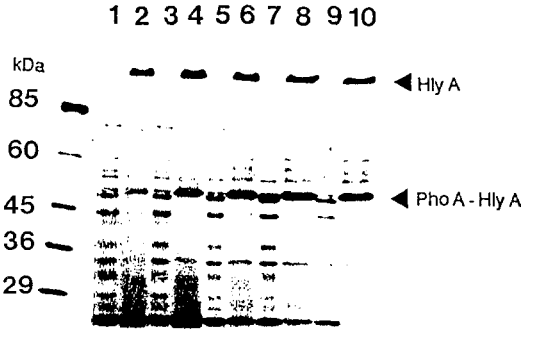

Fig. 5. Internal and external PhoA-HlyA$_s$ fusion protein expressed by *E. coli* CC118 carrying pJHH600B and pAL-C2 after induction with IPTG for 0.5 h (lanes 1, 2), 1 h (lanes 3, 4), 1.5 h (lanes 5, 6), 2 h (lanes 7, 8) and 2.5 h (lanes 9, 10). Lanes 2, 4, 6, 8 and 10 show external proteins; lanes 1, 3, 5, 7 and 9 the internal proteins. External protein was 100-fold concentrated compared to internal protein. Proteins were separated by SDS-PAGE and stained with silver. The positions of HlyA (110 kDa) and the PhoA-HlyA$_s$ fusion protein are indicated

fusion protein is secreted in the presence of HlyB/HlyD (provided by pAL-C2). The amount of secreted fusion protein is in both cases considerably higher (60%–70%) than that retained inside the cell (30%–40%), suggesting that transport of both fusion proteins by HlyB/HlyD is equally efficient. These data indicate that the last C-terminal 60 amino acids of HlyA contain all of the information for HlyB/HlyD-dependent protein transport. We

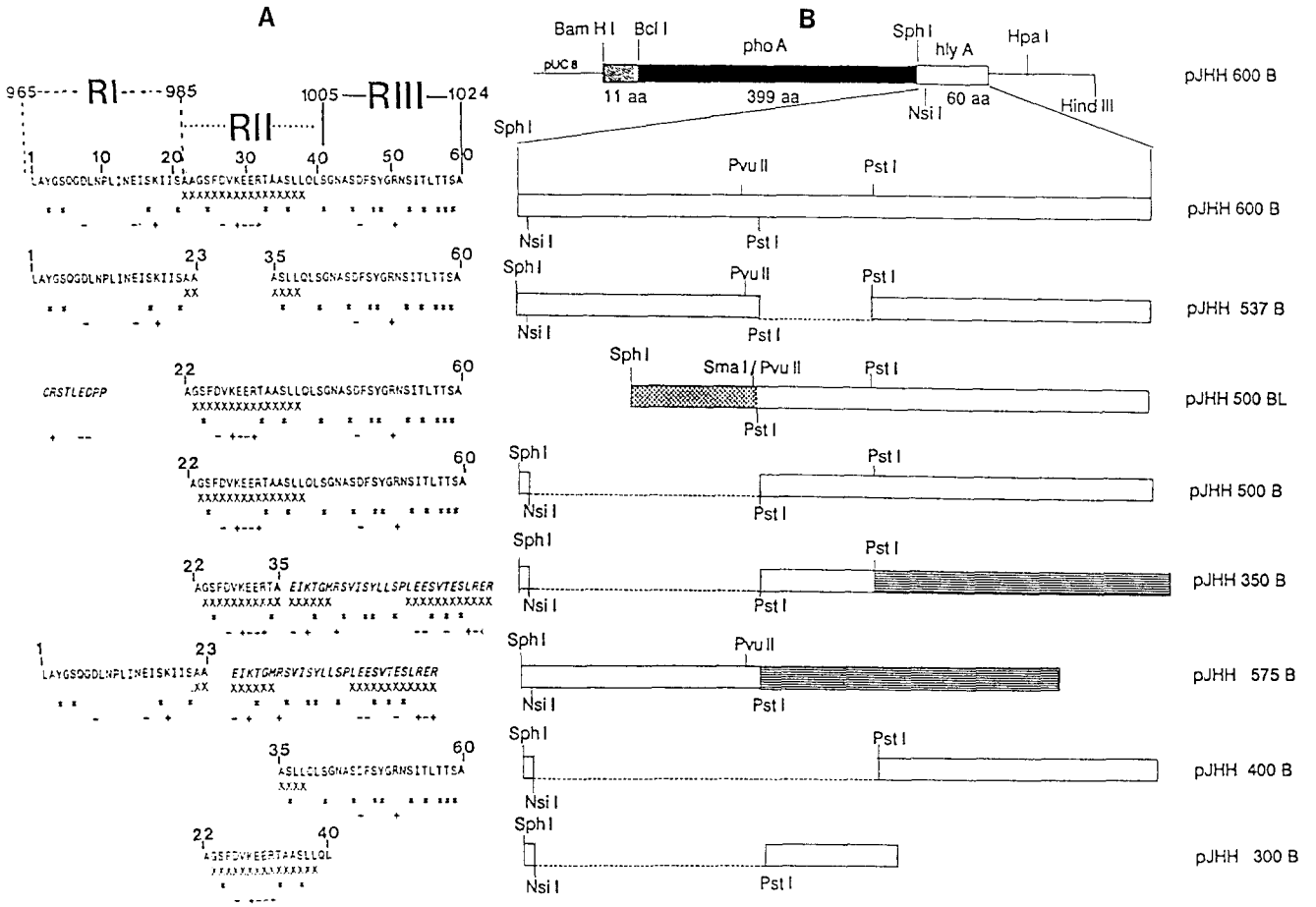

Fig. 6. A The C-terminal 60 amino acid peptide of HlyA and the deletions and substitutions introduced into this sequence. RI, RII and RIII indicate the three structural regions within this peptide. Sequence motifs are according to the predictions given by the algorithms of Garnier et al. (1978); xxx, α-helical regions; − and +, negatively or positively charged amino acids; *, hydroxylated amino acids. **B** Schematic presentation of the construction of the various mutants deriving from pJHH600B. *Dotted lines* indicate deletions, *shaded bars* represent substitutions by non-HlyA specific amino acids (indicated in *italics* in **A**) and *open bars* the retained HlyA specific sequences

shall designate this portion of HlyA, carrying the C-terminal 60 amino acids, as $HlyA_s$. Transport functions for PhoA-$HlyA_s$ can also be provided by pBD152 with a similar efficiency to pAL-C2 (data not shown). In contrast to pAL-C2, plasmid pBD152 does not express HlyA. It can therefore be excluded that the high transport efficiency of PhoA-$HlyA_s$ observed in the combination of plasmids pJHH600B and pAL-C2 is caused by a pickaback transport of the fusion protein with HlyA. To test possible competition between the PhoA-$HlyA_s$ fusion protein and HlyA, secretion of both proteins was studied in *E. coli* CC118 carrying pJHH600B and pAL-C2. The PhoA-$HlyA_s$ fusion protein was induced with 1 mM IPTG and secretion of both proteins was followed for 2.5 h after induction (Fig. 5). Whereas the amount of secreted PhoA-$HlyA_s$ is relatively low at the time of induction compared to that of HlyA, the amount of the fusion protein increases about tenfold 2 h after induction and exceeds that of HlyA. Even the secretion of this large amount of PhoA-$HlyA_s$ does not interfere with the secretion of HlyA, as the amount of HlyA secreted remains constant throughout the time of induction. Note that the level of intracellular PhoA-$HlyA_s$ is low, which suggests that the efficiency of secretion of the PhoA-$HlyA_s$ protein is high and close to that of the natural substrate HlyA, an internal pool of which cannot be detected.

Effects of deletions and substitutions within the 60 amino acid signal of HlyA suggest three separate functional regions

The 60 amino acid sequence of the signal peptide of HlyA is depicted in Fig. 6A. It represents amino acids 965–1024 of the HlyA sequence of pHly152 (Hess et al. 1986). For reasons of simplicity we shall designate the 60 amino acids number 1 (identical to amino acid 965) to number 60 (identical to amino acid 1024). Computer analysis of the 60 amino acid peptide predicts the following features:

1. A Sequence of mainly neutral and polar amino acids followed by a stretch of hydrophobic residues (amino acids 1–21, region I, RI).
2. A sequence (amino acids 22–40) which continues the hydrophobic sequence of RI and contains an excess of

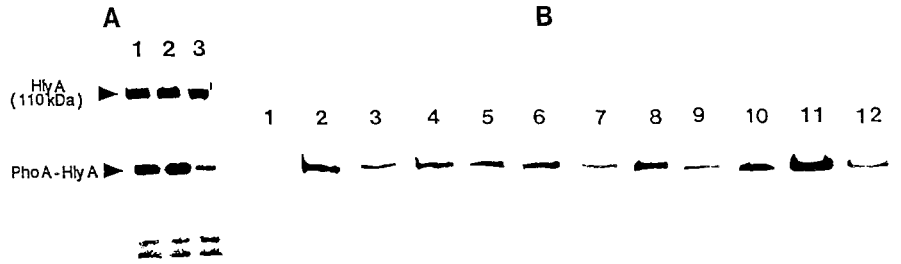

Fig. 7. A External PhoA-HlyA fusion proteins secreted by *E. coli* CC118 carrying pJHH500BL (lane 1), pJHH600B (lane 2) and pJHH500B (lane 3) complemented with pAL-C2. Proteins were separated by SDS-PAGE and stained with silver. The extracts loaded in each lane were prepared from equal numbers of bacterial cells. **B** Immunoblots showing internal (lanes 2, 4, 6, 8, 10, 12) and external (lanes 1, 3, 5, 7, 9, 11) PhoA-HlyA proteins expressed by *E. coli* CC118 carrying pJHH575B (lanes 1, 2), pJHH537B (lanes 3, 4), pJHH400B (lanes 5, 6), pJHH300B (lanes 7, 8) pJHH350B (lanes 9, 10) and pJHH600B as control (lanes 11, 12). Transport functions were provided in all cases by pAL-C2. The amounts of external protein loaded were prepared from 10 times as many cells as were used to assay internal protein except in the case pJHH600B where both extracts were derived from the same number of cells. Despite the different sizes of the HlyA signal peptides in the PhoA-HlyA fusion proteins, no differences in migration of these proteins were detectable under the conditions used

polar (mainly negatively charged) amino acids. This sequence is α-helical, has a low hydrophobic moment and is designated region II (RII).

3. A relatively hydrophobic sequence (amino acids 41–60; region III, RIII) contains at its end eight amino acids which may form a β-pleated sheet structure. This structural element contains mainly hydroxylated amino acids. A similar tripartite structure for this region of HlyA has been previously suggested for the last 53 amino acids of HlyA by Koronakis et al. (1989).

In order to analyse the functional importance of these three regions of the HlyA signal with respect to recognition by the HlyB/HlyD translocation system, deletions and substitutions were introduced into this sequence. The construction of these variants is schematically shown in Fig. 6B. All sequences were verified by DNA sequence analysis of the altered region. The altered signal sequences of the variants are summarized in Fig. 6A. The sequence of PhoA-HlyA500B has lost amino acids 1–23 which includes the entire region I. As shown in Fig. 7A this fusion protein is still secreted when the strain carrying pJHH500B is complemented with pAL-C2. The transport efficiency of this fusion protein relative to that of PhoA-HlyA600B (carrying the intact 60 amino acid HlyA signal) and the internal HlyA standard is, however, only 10%. The transport efficiency is considerably increased by the addition of a nonapeptide which contains two Pro residues in positions 8 and 9. This sequence, which is entirely unrelated in amino acid composition to the region I sequence, restores the secretion efficiency of the fusion protein PhoA-HlyA500BL to almost the same level as that of PhoA-HlyA600B (Fig. 7A). In PhoA-HlyA537B amino acids 23–34 are deleted. This deletion removes region II which includes a stretch of polar amino acids (DVKEER). As shown in Fig. 7B, this fusion protein is still secreted by HlyB/HlyD but the transport efficiency is decreased to about 1%–5% of that of PhoA-HlyA600B. In the PhoA fusion proteins encoded by the recombinant plasmids pJHH300B and pJHH350B, region II is retained whereas regions I and III are missing. PhoA-HlyA350B differs from PhoA-HlyA300B by the addition of 28 mainly α-helix-forming amino acids unrelated to HlyA at the C-terminal end. HlyB/HlyD-dependent secretion is still observed with both fusion proteins although the secretion efficiency is very low (about 0.1% of that of PhoA-HlyA600B in both cases). A slightly higher secretion efficiency (more than 1% of that of PhoA-HlyA600B) is observed with the fusion protein PhoA-HlyA400B which lacks regions I and II but retains region III. No HlyB/HlyD-specific secretion is obtained with PhoA-HlyA575B, which carries domain I but lacks domains II and III. This fusion protein contains, in addition, the same 28 amino acid sequence as PhoA-HlyA350B at the C-terminal end.

Discussion

The haemolysin secretion system requires, in addition to the two membrane proteins HlyB and HlyD, which together are thought to form a specific transmembrane pore, sequences in haemolysin (HlyA) which recognize this protein translocation system. There is now convincing evidence that the transport signal is located at the C-terminal end of HlyA (Härtlein et al. 1983; Nicaud et al. 1986; Koronakis et al. 1989; Mackman et al. 1987; Gentschev et al. 1990). The reported data dealing with the characterization of this HlyA sequence necessary for transport do not allow us clearly to define the size and the functionally essential parts of this sequence, nor do they exclude a requirement for additional sequences of HlyA besides the C-terminal region. Our approaches to the experimental analysis of these questions are twofold: (a) we used a fusion protein consisting of a reporter protein and the HlyA signal which is detectable in small amounts and stable under intracellular and extracellular conditions; and (b) an efficient *trans*-complementing secretion system which expresses HlyB and HlyD to such a level that the cellular concentration of this translocator does not become the rate-limiting step in the secretion of fusion proteins carrying the HlyA signal. We define

the HlyA signal sequence as the minimal HlyA sequence which will allow the secretion of a fusion protein by HlyB/HlyD with a similar efficiency to the natural substrate, which is the full-length HlyA polypeptide. We have found that a PhoA protein lacking its own N-terminal transport signal sequence and part of the C-terminus is well suited for this purpose. In a detailed study to define the minimal HlyA peptide still secreted by HlyB/HlyD, one of us (T. Jarchau) has recently shown that the smallest peptide that confers transport competence comprises the last 60 C-terminal amino acids of HlyA (T. Jarchau et al., manuscript in preparation). The PhoA-HlyA fusion protein constructed in this study takes these data into account. The results reported here demonstrate that fusion of this 60 amino acid HlyA sequence to PhoA yields a fusion protein which is secreted with an efficiency similar to that of a fusion protein containing a much larger segment of HlyA. This C-terminal part of the longer HlyA fusion includes the repeat region which has previously been postulated to be involved in HlyA secretion (Felmlee and Welch 1988). The present data clearly rule out this possibility in the case of fusion proteins. Furthermore, the secretion efficiency of the PhoA-HlyA$_s$ fusion protein carrying the 60 amino acid sequence is similar to that of HlyA itself. Based on these data we conclude that the last 60 amino acids of HlyA are necessary and sufficient for recognition of the HlyB/HlyD translocation system by the fusion proteins. It has been pointed out before (Koronakis et al. 1989) that this HlyA sequence contains structural features which divide it into three regions. Region I, comprising amino acids 1–21, is enriched in neutral polar amino acids. It has recently been claimed that this part is the most essential portion of the HlyA signal and consists of an amphiphilic α-helix (Koronakis et al. 1989). However, as assessed by the method of Eisenberg et al. (1982), this sequence does not possess a large hydrophobic moment because no extended helical structure can be formed in this region due to the presence of a Pro residue in position 11. Furthermore, our data with the PhoA-HlyA fusion proteins contradict the conclusion on the functional importance of region I. Although a PhoA fusion protein lacking this region (PhoA-HlyA500B) shows a dramatic decrease in secretion efficiency compared to that of PhoA-HlyA$_s$, a nonapeptide linker containing two Pro residues at the junction site to region II can almost completely replace region I. This nonapeptide has no amphiphilic character. These data suggest that the first region may represent a structure which exposes regions II and III at the end of the HlyA molecule. Furthermore, linkage of region I to PhoA (PhoA-HlyA575B) does not allow HlyB/HlyD-dependent secretion of this fusion protein. In contrast, fusion of region III (consisting of 20–22 relatively hydrophobic and hydroxylated amino acids to PhoA alone (PhoA-HlyA400B) or in combination with region I (PhoA-HlyA537B) allows HlyB/HlyD-dependent secretion of the fusion protein, albeit at low efficiency. Previous results have shown (Mackman et al. 1987) that no transport of N-terminal truncated HlyA protein is observed in the absence of region II. The reason for this discrepancy is currently unknown. Region II, which is predicted to form an α-helix and contains the most polar amino acids of the HlyA signal, can also confer a low secretion activity when fused to PhoA (PhoA-HlyA300B and PhoA-HlyA350B). The α-helical structure of this region does not seem to be of functional importance (Koronakis et al. 1989). Our data support the view that regions II and III recognize the HlyB/HlyD translocation system; presumably each domain can interact directly with the cytoplasmic portion of HlyB which appears to be sufficient for pulling HlyA into the membrane (Oropeza-Wekerle et al. 1990). Yet the low secretion efficiency obtained with these fusion proteins suggests that the interactions of the separate domains are weak and only the cooperative interaction of both domains with HlyB allows efficient binding of HlyA to the translocation system. The translocation system consisting of HlyB and HlyD has a high capacity for secreting suitable proteins when the two gene products are expressed at adequate levels. Interestingly, a recombinant plasmid carrying the *Bgl*II fragment from pHly152 containing both genes (*hlyB* and *hlyD*) cloned into the *tet* gene of pBR322 or pACYC184 was unable to complement transport of HlyA *in trans* when the protein concentration in the supernatants was assayed by gel electrophoresis. A similar clone, previously described by Mackman et al. (1987) carrying a *Bgl*II fragment of a chromosomal *hly* determinant has secretion-complementing activity (Nicaud et al. 1986). In contrast to the pHly152-derived *hly* determinant, chromosomal *hly* determinants do not seem to require the *cis*-activating element *hlyR* in order to express *hlyB* and *hlyD* (Welch and Pellet 1988). HlyR apparently causes antitermination of transcription at a transcriptional terminator sequence located behind *hlyA* (Koronakis et al. 1988). It appears that in pHly152-derived constructs containing this terminator sequence, antitermination can only be accomplished in the presence of *hlyR*, whereas possibly a host factor can perform this antitermination in the case of the chromosomal *hly* determinants. The two *hlyR* containing plasmids pAL-C2 and pBD152 express the HlyB/HlyD translocation system very efficiently. The fact that transport efficiency expressed by pBD152 with the described PhoA-HlyA$_s$ fusion protein is as high as that of pAL-C2 (which additionally expresses HlyA) rules out the possibility that a pickaback transport together with HlyA may facilitate the secretion of the fusion proteins. Even in the presence of large amounts of the fusion protein, HlyA is transported with normal efficiency, indicating that transport of the fusion protein does not compete with that of HlyA. These data suggest that even under these conditions, where several-fold more fusion protein molecules are secreted than the number of HlyA molecules normally translocated, the capacity of the HlyB/HlyD is not saturated.

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References

Baty D, Lloubes R, Geli V, Lazdunski C, Howard SP (1987) Extracellular release of colicin A is non-specific. EMBO J 6:2463–2468

Boyer HW, Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol 41:459–472

Carter-Muenchau P, Wolf RE Jr (1987) A method for cloning mixtures of long, synthetic oligonucleotides. Gen Anal Techn 4:105–110

Eisenberg D, Weiss RM, Terwilliger TC (1982) The helical hydrophobic moment: a measure of the amphiphilicity of a helix. Nature 299:371–374

Erb K, Vogel M, Wagner W, Goebel W (1987) Alkaline phosphatase which lacks its own signal sequence becomes enzymatically active when fused to N-terminal sequences of *Escherichia coli* haemolysin (HlyA). Mol Gen Genet 208:88–93

Felmlee T, Welch RA (1988) Alterations of amino acid repeats in the *Escherichia coli* hemolysin affect cytolytic activity and secretion. Proc Natl Acad Sci USA 85:5269–5273

Garnier J, Osguthorpe DJ, Robson B (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 120:97–120

Gentschev I, Hess J, Goebel W (1990) Change in the cellular localization of alkaline phosphatase by alteration of its carboxy-terminal sequence. Mol Gen Genet 222:211–216

Goebel W, Hedgpeth J (1982) Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli*. J Bacteriol 151:1290–1298

Härtlein M, Schießl S, Wagner W, Rdest U, Kreft J, Goebel W (1983) Transport of hemolysin by *Escherichia coli*. J Cell Biochem 22:87–97

Hess J (1990) Transport von Hämolysin in *Escherichia coli*. PhD thesis, University Würzburg, FRG

Hess J, Wels W, Vogel M, Goebel W (1986) Nucleotide sequence of a plasmid encoded hemolysin determinant and its comparison with a corresponding chromosomal hemolysin sequence. FEMS Microbiol Lett 34:1–11

Holland IB, Wang R, Seror SJ, Blight M (1989) Haemolysin secretion and other protein translocation mechanisms in gram-negative bacteria. In: Baumberg S, Hinster I, Rhodes M (eds) Microbial Products, New Approaches. Cambridge University Press, Cambridge, pp 219–254

Koronakis V, Cross M, Hughes C (1988) Expression of the *E. coli* hemolysin secretion gene *hly*B involves transcript anti-termination within the *hly* operon. Nucleic Acids Res 16:4789–4800

Koronakis V, Koronakis E, Hughes C (1989) Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin across both bacterial membranes. EMBO J 8:595–605

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. Nature 227:680–685

Lee C, Beckwith J (1986) Cotranslational and post-translational protein translocation in procaryotic systems. Annu Rev Cell Biol 2:315–336

Ludwig A, Vogel M, Goebel W (1987) Mutations affecting activity and transport of haemolysin in *E. coli*. Mol Gen Genet 206:238–245

Mackman N, Nicaud JM, Gray L, Holland IB (1984) Functional characterization of a cloned haemolysin determinant from *E. coli* of human origin, encoding information for the secretion of a 107 K polypeptide. Mol Gen Genet 196:129–134

Mackman N, Baker K, Gray L, Haigh R, Nicaud JM, Holland B (1987) Release of a chimeric protein into the medium from *Escherichia coli* using the C-terminal secretion signal of haemolysin. EMBO J 6:2835–2841

Manoil C, Beckwith J (1985) TnPhoA: A transposon probe for protein export signals. Proc Natl Acad Sci USA 82:8129–8133

Nicaud JM, Mackman N, Gray L, Holland IB (1986) The C-terminal 23 kD peptide of *Escherichia coli* haemolysin 2001 contains all the information necessary for its secretion by the haemolysin (Hly) machinery. FEBS Lett 204:331–335

Oakley BR, Kirsch DR, Morris NR (1980) A simplified silver stain for detecting proteins in polyacrylamide gels. Anal Biochem 105:361–363

Oliver D, Beckwith J (1981) *E. coli* mutant pleiotropically defective in the export of secreted proteins. Cell 25:765–772

Oropeza-Wekerle RL, Müller E, Kern P, Meyermann R, Goebel W (1989) Synthesis, inactivation and localization of extracellular and intracellular *Escherichia coli* hemolysins. J Bacteriol 171:2783–2788

Oropeza-Wekerle RL, Speth W, Imhof B, Gentschev I, Goebel W (1990) Translocation and compartmentalization of *Escherichia coli* hemolysin (HlyA). J Bacteriol 172:3711–3717

Pohlner J, Halter R, Beyreuther K, Meyer TF (1987) Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. Nature 325:458–462

Pugsley AP, Reyss I (1990) Five genes at the 3′ end of the *Klebsiella pneumoniae pul*C operon are required for pullulanase secretion. Mol Microbiol, in press

Pugsley AP, Schwartz M (1985) Export and secretion of proteins by bacteria. FEMS Microbiol Rev 32:3–38

Randall LL, Hardy SJS, Thom JR (1987) Export of proteins: a biochemical view. Annu Rev Microbiol 41:507–541

Saier MH, Werner PK, Müller M (1989) Insertion of proteins into bacterial membranes: mechanism, characteristics and comparisons with the eucaryotic process. Microbiol Rev 53:333–366

Towbin H, Staehlin H, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Vogel M, Hess J, Then I, Juarez A, Goebel W (1988) Characterization of a sequence (hlyR) which enhances synthesis and secretion of hemolysin in *Escherichia coli*. Mol Gen Genet 212:76–84

Wagner W, Vogel M, Goebel W (1983) Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. J Bacteriol 154:200–210

Walter P, Lingappa VR (1986) Mechanisms of protein translocation across the endoplasmic reticulum membrane. Annu Rev Cell Biol 2:499–516

Welch RA, Pellett S (1988) The transcriptional organization of the *Escherichia coli* hemolysin genes. J Bacteriol 170:1622–1630

Wolfe PB, Rice M, Wickner W (1985) Effects of two *sec* genes on protein assembly into the plasma membrane of *Escherichia coli*. J Biol Chem 260:1836–1841

Yanagida N, Uuzumi T, Beppu T (1986) Specific excretion of *Serratia marcescens* protease through the outer membrane of *E. coli*. J Bacteriol 166:937–944

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