Properties of haemolysin E (HlyE) from a pathogenic *Escherichia coli* avian isolate and studies of HlyE export

Neil R. Wyborn,1 Angela Clark,1 Ruth E. Roberts,1 Stuart J. Jamieson,1 Svetomir Tzokov,1 Per A. Bullough,1 Timothy J. Stillman,1 Peter J. Artymiuk,1 James E. Galen,2 Licheng Zhao,2 Myron M. Levine2 and Jeffrey Green1

1Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2TN, UK
2Center for Vaccine Development, Division of Infectious Diseases and Tropical Pediatrics, University of Maryland School of Medicine, 685 W. Baltimore St, Baltimore, MD 21201, USA

Haemolysin E (HlyE) is a novel pore-forming toxin first identified in *Escherichia coli* K-12. Analysis of the 3-D structure of HlyE led to the proposal that a unique hydrophobic β-hairpin structure (the β-tongue, residues 177–203) interacts with the lipid bilayer in target membranes. In seeming contradiction to this, the *hlyE* sequence from a pathogenic *E. coli* strain (JM4660) that lacks all other haemolysins has been reported to encode an Arg residue at position 188 that was difficult to reconcile with the proposed role of the β-tongue. Here it is shown that the JM4660 *hlyE* sequence encodes Gly, not Arg, at position 188 and that substitution of Gly188 by Arg in *E. coli* K-12 HlyE abolishes activity, emphasizing the importance of the head domain in HlyE function. Nevertheless, 76 other amino acid substitutions were confirmed compared to the HlyE protein of *E. coli* K-12. The JM4660 HlyE protein was dimeric, suggesting a mechanism for improving toxin solubility, and it lysed red blood cells from many species by forming 36–41 Å diameter pores. However, the haemolytic phenotype of JM4660 was found to be unstable due to defects in HlyE export, indicating that export of active HlyE is not an intrinsic property of the protein but requires additional components. TnphoA mutagenesis of *hlyE* shows that secretion from the cytoplasm to the periplasm does not require the carboxyl-terminal region of HlyE. Finally, disruption of genes associated with cell envelope function, including *tatC*, impairs HlyE export, indicating that outer membrane integrity is important for effective HlyE secretion.

INTRODUCTION

Recently a novel pore-forming toxin designated HlyE, ClyA or SheA has been identified in *Escherichia coli* and in *Salmonella enterica* serovars Typhi and Paratyphi A (del Castillo et al., 1997; Green & Baldwin, 1997; Oscarsson et al., 1996, 2002; Wallace et al., 2000). The HlyE protein is a 34 kDa rod-shaped molecule consisting of a bundle of four long (80–90 Å; 8–9 nm) helices, which coil around each other with significant elaborations at both poles of the four-helix bundle (Fig. 1) (Wallace et al., 2000). At the end that contains the N-terminal region an additional shorter (30 Å) helix (αC) packs against the four long helices, forming a five-helix bundle for about one-third of the length of the molecule (the tail domain). Mutagenesis has suggested that the αG region is involved in HlyE activity (Atkins et al., 2000; Oscarsson et al., 1999). The tail domain also houses the two Cys residues of HlyE, which can be linked by a disulphide bond (Atkins et al., 2000). At the opposite end of the molecule there is a subdomain (the head domain) consisting of a short two-stranded antiparallel β-sheet flanked by two short helices (the β-tongue), located between the third and fourth helices of the main bundle (Wallace et al., 2000). The hydrophobic nature of the β-tongue has to be maintained to allow interaction between HlyE and target membranes (Oscarsson et al., 1999; Wallace et al., 2000). The HlyE protein forms pores in target membranes that appear in electron microscope images as ring-shaped structures with an internal diameter of 42–52 Å, when viewed from above, and as 100–105 Å spikes in side view, suggesting that HlyE does not undergo large conformational changes during pore formation (Wallace et al., 2000).

In *E. coli* K-12, *hlyE* expression is activated by the action of the global transcription factors FNR and CRP (cAMP receptor protein), in response to oxygen starvation and glucose starvation, respectively (Green & Baldwin, 1997; Wall...
Westernmark et al., 2000). Thus, expression of this gene responds to environmental conditions related to infection. E. coli is a major pathogen of birds, where infection usually begins with respiratory disease followed by septicaemia, granuloma, peritonitis, salpingitis, omphalitis and airsacsculitis (Gross, 1991). Such pathological conditions lead to significant economic losses in the production of chickens and turkeys. Several protein toxins associated with E. coli pathogenesis have been identified, including haemolysins. The production of haemolysins by E. coli is associated with extra-intestinal infections in man, and thus haemolysins are considered to play an important role in pathogenesis. Indeed, the HlyE protein has been shown to be cytotoxic and apoptogenic to human and murine monocytes (Lai et al., 2000). The hlyE gene from a pathogenic E. coli avian strain, JM4660, that lacks the uropathogenic and enterohaemorrhagic haemolysin gene, hlyD, was isolated and sequenced (Reingold et al., 1999). According to this report, in comparison to E. coli K-12 the JM4660 hlyE gene encoded an amino acid substitution, Gly188→Arg (Reingold et al., 1999). Such a replacement was difficult to accommodate within the 3-D structure of HlyE without disrupting and rearranging the structure of the hydrophobic β-tongue of HlyE (Fig. 1) (Wallace et al., 2000). In view of its probable role as the major pore-forming toxin of E. coli JM4660 the properties of this protein were investigated.

**METHODS**

**Bacterial strains, plasmids and oligonucleotides.** These are listed in Table 1.

**PCR-based site-directed mutagenesis.** Site-directed mutagenesis was achieved by the overlap extension PCR technique (Sambrook & Russell, 2001) using appropriate mutagenic and flanking primers (Table 1). The authenticity of the altered hlyE genes was confirmed by automated DNA cycle sequencing before and after ligation into the expression vectors pTac85 (Marsh, 1986) and pGEX-KG (Guan & Dixon, 1991).

**Characterization of HlyE proteins.** The HlyE proteins were isolated from GST–HlyE-overproducing strains as previously described (Atkins et al., 2000). The haemolysis assay of Rowe & Welch (1994) with horse blood was used for routine measurement of HlyE activity. Blood from other species was tested using the same protocol. All blood samples were obtained from TCS Microbiology (UK). Mass spectrometry (Micromass MALDI-TOF) measurements were made using samples of HlyE that had been dialysed against deionized water. Native molecular masses were estimated by gel filtration on a calibrated Superdex 200 HR 10/30 column (Amersham) equilibrated with 50 mM sodium phosphate pH 7-0, containing 150 mM NaCl. The SDS-PAGE analysis of purified proteins was as described previously (Atkins et al., 2000). The thermostability of the HlyE proteins was estimated by incubating equivalent amounts of protein (0-1 mg ml⁻¹) for different periods at 37, 50 or 65 °C before incubating on ice for 30 min prior to determining the amount of HlyE activity remaining. The HlyE pores were visualized in lipid vesicles and the negatively stained images were obtained using the equipment and procedures previously described (Wallace et al., 2000).

**Distribution of HlyE.** Subcellular fractionation was achieved by collecting the bacteria from aerobic cultures [200 ml Lennox broth (tryptone, 10 g l⁻¹; yeast extract, 5 g l⁻¹; NaCl, 5 g l⁻¹)] in 2 l conical flasks, grown for 16 h at 37 °C with shaking, 250 r.p.m. of haemolytic and non-haemolytic variants of JM4660. The culture supernatants and bacterial pellets were retained. The supernatants were passed through 0.2 μm filters to remove any remaining bacteria before analysis, unless indicated. The bacterial cells were fractionated according to the protocol described by Sambrook & Russell (2001). The bacterial pellets were suspended in 20 mM Tris/ HCl, pH 8.0, containing 1-7 mM EDTA and 0-5 M sucrose (10 ml) and incubated for 1 h at room temperature with gentle shaking. The bacteria were then collected by centrifugation at 3500 g for 60 min at room temperature. The supernatants were discarded and the pellets resuspended in 10 mM Tris/HCl, pH 8-0 (4 ml). After incubation at room temperature with gentle shaking for 1 h the suspensions were centrifuged at 11 000 g for 30 min at 4 °C. The supernatant fraction contained periplasmic proteins (generally 0-8–1-0 mg protein ml⁻¹). Cytoplasmic fractions were then obtained from the pellets by sonication. The cytoplasmic fractions contained 0.9–1.3 mg protein ml⁻¹. The HlyE activities of the cytoplasmic and periplasmic fractions were expressed as the change in AS₄₃ min⁻¹ mg⁻¹, and for the external medium as the change in AS₄₃ min⁻¹ ml⁻¹ (Rowe & Welch, 1994). Aconitase and lipoamide dehydrogenase (E3 subunit of the pyruvate dehydrogenase complex) served as markers for the cytoplasmic fractions, and activities were assayed according to Gruer et al. (1997) and Creaghan & Guest (1972), respectively. Aconitase activity was expressed as nmol cis-aconitate produced min⁻¹ mg⁻¹ and lipoamide dehydrogenase activity as nmol APAD (acyetylpyridine dinucleotide) reduced h⁻¹ mg⁻¹.

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**Fig. 1.** The fold of the pore-forming toxin HlyE. The overall fold of an E. coli K-12 HlyE monomer is shown as a main chain ribbon with helices coloured cyan and strands coloured green. Non-identical residues between E. coli K-12 HlyE and avian E. coli HlyE are highlighted in dark blue and the positions of the conserved Cys residues (87 and 285) are shown in yellow. Secondary structural elements are labelled together with both the conserved Cys residues (87 and 285) are shown in yellow. Figure produced using MIDAS (Ferrin et al., 1988).
Table 1. Bacterial strains, plasmids and oligonucleotides

For the oligonucleotides listed, unique restriction sites are italicized and mismatched bases directing desired codon changes are underlined. ApR: ampicillin resistance; KanR: kanamycin resistance.

<table>
<thead>
<tr>
<th>Strain, plasmid or oligonucleotide</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli MC1000</td>
<td>K-12 strain that is normally non-haemolytic on blood agar</td>
<td>Silhavy et al. (1984)</td>
</tr>
<tr>
<td>E. coli JM4660</td>
<td>A haemolytic strain isolated from chicken</td>
<td>Reingold et al. (1999)</td>
</tr>
<tr>
<td>E. coli EC100</td>
<td>TransforMax EC100 Electrocompetent E. coli</td>
<td>Cambio</td>
</tr>
<tr>
<td>E. coli CC118</td>
<td>phoA20</td>
<td>Manoil &amp; Beckwith (1985)</td>
</tr>
<tr>
<td>E. coli SM10(pRT733)</td>
<td>thi thr leu tonA lacY supE recA::RP4-2-Tn::Mu</td>
<td>Taylor et al. (1989)</td>
</tr>
<tr>
<td>pGEX-KG</td>
<td>Expression vector with an IPTG-inducible promoter; ApR</td>
<td>Guan &amp; Dixon (1991)</td>
</tr>
<tr>
<td>pGS415</td>
<td>pGEX-KG derivative for expression of A. pleuropneumoniae hlyX; activates expression of hlyE and confers a haemolytic phenotype on EC100; ApR</td>
<td>Atkins et al. (2000)</td>
</tr>
<tr>
<td>pGS1111</td>
<td>pGEX-KG derivative for expression of E. coli K-12 hlyE as an N-terminal GST fusion protein; ApR</td>
<td>Atkins et al. (2000)</td>
</tr>
<tr>
<td>pGS1148</td>
<td>pGEX-KG derivative for expression of E. coli K-12 hlyE as an N-terminal GST fusion protein; ApR</td>
<td>This work</td>
</tr>
<tr>
<td>pGS1360</td>
<td>pGEX-KG derivative for expression of E. coli K-12 hlyE(Ala187→Gly, Gly188→Arg) variant; ApR</td>
<td>This work</td>
</tr>
<tr>
<td>pGS1361</td>
<td>pGEX-KG derivative for expression of E. coli JM4660 hlyE; ApR</td>
<td>This work</td>
</tr>
<tr>
<td>pGS1590</td>
<td>pGEX-KG derivative for expression of E. coli JM4660 hlyE as an N-terminal GST fusion protein; ApR</td>
<td>This work</td>
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<td>pGS1591</td>
<td>pGEX-KG derivative for expression of E. coli JM4660 hlyE as an N-terminal GST fusion protein; ApR</td>
<td>This work</td>
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<tr>
<td>pGS1699</td>
<td>pBAD-HisA (Invitrogen) derivative carrying hlyE-srrA</td>
<td>This work</td>
</tr>
<tr>
<td>EZ::Tn &lt;KAN-2&gt;Tnp</td>
<td>Transposome; KanR</td>
<td>Cambio</td>
</tr>
<tr>
<td>AA32</td>
<td>TAATCC AAAATTGCCGCGCGACGACACC (reverse primer directing Ala187→Gly, Gly188→Arg in E. coli K-12 HlyE)</td>
<td>This work</td>
</tr>
<tr>
<td>AA33</td>
<td>AGGCCGTTGCGTGGCGCGTCCATTTGG (forward primer directing Ala187→Gly, Gly188→Arg in E. coli K-12 HlyE)</td>
<td>This work</td>
</tr>
<tr>
<td>JGC1</td>
<td>GAGGCCGATGACCATGCTGGTAAATCGC (forward primer for amplification of the E. coli K-12 hlyE)</td>
<td>Atkins et al. (2000)</td>
</tr>
<tr>
<td>JGD1</td>
<td>CACACGGTAACTGTGCAACCTTTAAAAACCGTG (reverse primer for amplification of the E. coli K-12 hlyE)</td>
<td>Atkins et al. (2000)</td>
</tr>
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<td>AA49</td>
<td>TTTTGGATCCATGACCAATGAGCATGACGTTGAACACAG (forward primer for amplification of JM4660 hlyE)</td>
<td>This work</td>
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<td>AA50</td>
<td>TTTTGCGAAGATTAATACCAAGAGGGTTGCTTATTCTTCCC (reverse primer for amplification of JM4660 hlyE)</td>
<td>This work</td>
</tr>
<tr>
<td>AA53–64</td>
<td>A series of oligonucleotides within the hlyE coding region for determining the sequence of the avian hlyE genes</td>
<td>This work</td>
</tr>
</tbody>
</table>

Mutagenesis. The transposon mutagenesis strategy used was based on the Epicentre EZ::Tn< KAN-2 > Tnp transposome kit (Cambio). The electrocompetent E. coli strain EC100 (>1 × 10^8cfu.ml⁻¹; supplied by Cambio) was simultaneously transformed with pGS415 (Table 1) and the transposome complex EZ::Tn< KAN-2 > Tnp (Table 1). After outgrowth for 1 h at 37°C, transformants were plated on blood agar containing ampicillin (100 µg ml⁻¹), kanamycin (30 µg ml⁻¹) and IPTG (0.125 mM) to yield ~150–200 colonies per plate. After growth at 37°C for 16–24 h, colonies displaying reduced haemolysis were selected and streaked onto fresh plates to confirm the phenotype. Genomic DNA was then prepared from each isolate using the Qiagen DNeasy protocol. Direct genomic DNA sequencing, using the primer FP-1 (ACCTA CAAAGAAGCTTCTCATCAACCCambio), which is complementary to the EZ::Tn< KAN-2 > Tnp sequence, was used to locate the transposon insertion site within the genome. Specifically, template DNA was incubated at 55°C for 30 min, to enhance solubility, before vortexing to shear the DNA. The sheared DNA (3–5 µg per reaction) was incubated at 95°C for 5 min in the presence of the primer (FP-1) and Thermofidelase I (1 µl per reaction; Fidelity Systems), to unwind the template DNA. The Big Dye terminator mix (8 µl per reaction; ABI Prism) was then added along with distilled H₂O to a final volume of 40 µl. Following 99 cycles of strand separation (95°C, 30 s) and annealing/primer extension (60°C, 4 min) the products were purified using the DyeEx 2.0 spin protocol (Qiagen) and separated on an ABI Prism 377 DNA sequencer. Disrupted genes were identified using the Colibri (http://genolist.pasteur.fr/Colibri/index.html) or NCBI (http://www.ncbi.nlm.nih.gov/) websites.

The TnpA mutagenesis was done with transformants of E. coli HlyE from a pathogenic E. coli avian isolate
DH5x containing a plasmid (pGEM-ThyE) that expresses a functional S. enterica Serovar Typhi haemolysin E. After cross-streak mating between DH5x(pGEM-ThyE) and the TnphoA donor strain SM10(pRl733), transconjugants were selected on 2 x LB50 (tryptone, 20 g l\(^{-1}\); yeast extract, 10 g l\(^{-1}\); NaCl, 5 g l\(^{-1}\)) supplemented with tetracycline (10 µg ml\(^{-1}\)), carbenicillin (50 (µg ml\(^{-1}\)) and kanamycin (10 µg ml\(^{-1}\)). Pooled bacteria were grown and plasmids recovered for transformation into E. coli CC118 (phoΔ220) for selection of Pho\(^{-}\) transformants in the presence of the antibiotics listed above and the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate. Because the C-terminal region of the target protein is replaced by PhoA this method is unable to detect those proteins whose secretion is directed by C-terminally encoded signals.

**RESULTS**

**The hlyE gene of E. coli JM4660 does not encode Arg at position 188**

The starting point for this work was the report that a strain of *E. coli* (JM4660) isolated from a chicken with colibacillosis and airsacculitis lacked the uropathogenic *E. coli* strain of *E. coli* K-12 haemolysin E gene (Reingold et al., 1999). The JM4660 hlyE gene encoded a number of amino acid substitutions when compared to the *E. coli* K-12 sequence (Reingold et al., 1999). One of these substitutions, the replacement of Gly188 by Arg (numbering as for *E. coli* K-12) was problematical in the context of the *E. coli* K-12 HlyE 3-D structure (Wallace et al., 2000). This residue is located just before the β-turn in the β-tongue region, which forms the major hydrophobic patch of the HlyE surface and is responsible for interaction of HlyE with target membranes (Fig. 1).

![Fig. 2. Sequence alignments of members of the HlyE toxin family. The amino acid sequences of the HlyE toxin family studied in this work are shown: K-12, *E. coli* K-12; JM4660, avian *E. coli* isolate, as determined by Reingold et al. (1999); JM4660, the revised JM4660 sequence reported here. The *E. coli* K-12 sequence is used as the reference sequence, and where the other bacterial sequences differ from this is the amino acid shown. Every tenth residue is marked, and the α helices (*) and β strands (–1–; –2–) are indicated above the sequence. The Arg residue at position 188 in the original JM4660 sequence is highlighted in bold and underlined.](image-url)
occupied by these strains, the JM4660 HlyE protein was isolated after overproduction from pGS1591 as a GST–HlyE fusion protein as described previously for the *E. coli* K-12 HlyE protein (Atkins et al., 2000). As a consequence of the strategy used to isolate the JM4660 protein, it, like the *E. coli* K-12 HlyE, was expected to possess an additional 15 N-terminal amino acids encoded by the thrombin-sensitive linker of pGEX-KG. Confirmation of the presence of these additional amino acids (shown in italics, see below) and that the JM4660 HlyE had been overproduced was provided by N-terminal amino acid sequencing (GSPGISGGGGGILDSMTNADQTVETVKTAID). Despite the differences in primary structure between the K-12 and JM4660 HlyE proteins they proved to have very similar properties. The JM4660 protein formed pores in lipid vesicles, which when viewed perpendicular to the plane of the membrane appeared as clustered ring-shaped assemblies with an internal diameter of 36–41 Å, and external diameters of 86–92 Å (Fig. 3). These structures resembled the majority of the pores formed by the *E. coli* K-12 HlyE protein (42–52 Å internal diameter and 70–90 Å external diameter; Wallace et al., 2000). The JM4660 HlyE pores were homogeneous, and examples of the larger assemblies (55–60 Å internal diameter and 90–105 Å external diameter; Wallace et al., 2000) that form a minority population amongst the K-12 HlyE pores were not observed with JM4660 protein (Table 2). Thus, the dimensions of the JM4660 assemblies are consistent with the formation of an octomeric pore (Wallace et al., 2000; Atkins et al., 2000). Some images contained a central, stain-excluding density suggesting that, in these cases, the diameter of the pore might be restricted (Fig. 3). In addition, side-views of the pore assemblies could be seen as spikes (90–100 Å) extending from the edges of the vesicles, indicating that, as observed with the *E. coli* K-12 protein (Wallace et al., 2000), major conformational changes are not required for HlyE to interact with target membranes. Moreover, the JM4660 HlyE was resistant to protease treatment, was inhibited by incubation with Hg(II), and did not require post-translational modification for activity (Table 2). Estimation of the native molecular mass of JM4660 HlyE indicated that in contrast to the K-12 HlyE, the majority of the protein was dimeric (Table 2). Like *E. coli* K-12 HlyE, the protein from JM4660 was active against red blood cells from a wide range of species. However, it may be significant, in view of its avian niche, that it was twice as effective as the K-12 protein against chicken erythrocytes (Table 3), and that it had enhanced thermostability compared to the K-12 protein, in accord with the higher core temperature of birds compared to mammals (Table 2). These adaptations may be mediated by the many amino acid sequence differences compared to the K-12 protein, which presumably contribute to the predominance of the dimeric form of the JM4660 protein.

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**Table 2.** Properties of HlyE proteins isolated from GST–HlyE fusions

<table>
<thead>
<tr>
<th>Property</th>
<th>Source of HlyE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted molecular mass (Da)</td>
<td>K-12</td>
</tr>
<tr>
<td>Mass spectrometry (Da)</td>
<td>34 940</td>
</tr>
<tr>
<td>Subunit molecular mass (SDS-PAGE) (kDa)</td>
<td>34 476</td>
</tr>
<tr>
<td>Native molecular mass (gel filtration) (kDa)</td>
<td>&gt;300, 60 and 33</td>
</tr>
<tr>
<td>Half life at 50 °C (min)</td>
<td>5-0-5-5</td>
</tr>
<tr>
<td>Activity remaining after treatment with 5 mM Hg(II)</td>
<td>2-5%</td>
</tr>
<tr>
<td>Activity remaining after treatment with 600 U trypsin</td>
<td>100%</td>
</tr>
<tr>
<td>Inner diameter of pore (Å)</td>
<td>42–52 (major group) and 55–60 (minor group)</td>
</tr>
</tbody>
</table>
Table 3. Activity of isolated HlyE proteins from E. coli K-12 and JM4660 with different target erythrocytes

<table>
<thead>
<tr>
<th>Source of red blood cells</th>
<th>K-12 HlyE</th>
<th>JM4660 HlyE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sheep</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>Rabbit</td>
<td>33</td>
<td>11</td>
</tr>
<tr>
<td>Guinea pig</td>
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<td>28</td>
</tr>
<tr>
<td>Chicken</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Goose</td>
<td>46</td>
<td>49</td>
</tr>
</tbody>
</table>

Haemolytic activity was determined as described in Methods with equivalent amounts (20 ng) of HlyE protein from the indicated source. The values given are the means of duplicate assays from two experiments that varied by <10 %. 100 % is equivalent to a specific activity of 4 x 10^5 units mg^−1.

During the isolation of the JM4660 HlyE it was noted that much of the GST–HlyE protein bound to GSH-Sepharose was associated with lipid, appearing as a brown band at the top of the chromatography columns. This complex was released from the chromatography medium upon treatment with thrombin, suggesting that the GST component of the fusion was free to interact with the GSH-Sepharose and thrombin, and that the HlyE component was associated with the E. coli lipids. However, this was not observed with the E. coli K-12 GST-HlyE fusion. The protein profile of the thrombin-released JM4660 HlyE–lipid complexes was analysed by N-terminal amino acid sequencing. Three major polypeptides were visible on Coomassie-blue-stained denaturing gels. The major band (49 % of total protein in the sample) was HlyE (GSPGISGG). The second most abundant polypeptide (17 % of the total) had an apparent molecular mass and amino acid sequence (AEVYNKD) identical to OmpC, OmpN and YedS. The third most abundant polypeptide (11 % of the total) identified was SdhB (MRLEFSIY), the iron–sulphur protein component of the inner-membrane-bound succinate dehydrogenase complex. This suggests that the JM4660 HlyE is associated with E. coli membranes under these conditions, but conversely, such an association was not apparent for the K-12 HlyE. Evidence that HlyE can interact with E. coli lipids, forming assemblies resembling those observed for eukaryotic lipid vesicles, was obtained by electron microscopy (not shown). Although the interaction with the E. coli lipids occurred much more slowly than equivalent reactions with eukaryotic lipids, the physical appearance of the pores was indistinguishable. These observations suggest that the amino acid changes in JM4660 HlyE affect the cellular location of this protein in E. coli, which ultimately may have consequences for its action on target cells.

Status of the hlyE genes of non-haemolytic variants of E. coli JM4660

During the isolation of the JM4660 hlyE gene it was noticed that after overnight growth on blood agar a significant number of colonies with an impaired haemolytic phenotype were present. Picking and restreaking haemolytic colonies always yielded some apparently non-haemolytic colonies amongst the majority of strongly haemolytic colonies (Fig. 4). In aerobic liquid culture in rich medium (Lennox broth) the non-haemolytic variants grew as well as their haemolytic parents, and so the non-haemolytic phenotype was apparently not caused by a general growth defect (not shown). These observations were interesting because previous reports have indicated that the hlyE genes of many clinical strains of E. coli contain deletions that preclude the formation of functional HlyE protein (Atkins et al., 2000; Ludwig et al., 1999). Therefore, high-fidelity PCR (using primers AA49 and AA50, see Table 1) was used to amplify and isolate the hlyE genes from one haemolytic (H) and four non-haemolytic colonies of JM4660 (designated J1–J4). The PCR products were then sequenced by automated cycle DNA sequencing with primers AA53–64 (Table 1). Surprisingly, all the non-haemolytic isolates (J1–J4) tested still possessed full-length hlyE genes, predicted to encode proteins with amino acid sequences identical to that of the haemolytic strain. Thus, it would appear that the non-haemolytic variants of JM4660 had arisen by mechanisms other than by mutations within the hlyE gene.

Non-haemolytic variants of E. coli JM4660 fail to export HlyE

Because the four apparently non-haemolytic JM4660 variants (J1–J4) all possessed the same complete and unaltered hlyE coding regions, their ability to produce active HlyE protein was tested. This was done to establish whether these bacteria had become non-haemolytic through mutations affecting transcription or translation. Cell-free extracts from aerobic overnight cultures of the five JM4660 isolates (one haemolytic, H, and the four non-haemolytic isolates, J1–J4) were tested for haemolytic activity. As expected the cell-free extracts prepared from the haemolytic strain were active against horse erythrocytes, but surprisingly the cell-free extracts from the non-haemolytic variants (J1–J4) also possessed haemolytic activity (Table 4). Therefore, it was concluded that the non-haemolytic phenotype was not due to an inability to produce the HlyE protein per se.
The export of HlyE from *E. coli* is poorly understood. The protein itself does not possess any of the previously recognized signal sequences that target a protein for export. However, it is known that when HlyE is overproduced in *E. coli* K-12 the protein accumulates in the periplasm, and thus there must be a mechanism for trafficking HlyE to the periplasmic space. It has been suggested that blebbing of outer membrane vesicles provides at least one mechanism by which HlyE is released from the periplasm into the external medium (Wai et al., 2000, 2003a). Therefore, subcellular fractionation was used to isolate cytoplasmic, periplasmic and extracellular fractions from overnight aerobic cultures of the five JM4660 isolates (one haemolytic, H, and four non-haemolytic colonies, J1–J4, shown in Fig. 4). Activity measurements revealed that the HlyE protein was present in all three fractions (extracellular, cytoplasmic and periplasmic) obtained from the haemolytic strain. However, although HlyE activity was present in the cytoplasmic fractions it was not detected in the periplasm or extracellular medium of the non-haemolytic variants (Table 4). In two cases (J2 and J3) the HlyE activities of the cell-free extracts were significantly lower than those of the other strains (H, J1 and J4), suggesting that less HlyE protein is produced by these strains (Table 4). The HlyE activities in cell-free extracts of the remaining non-haemolytic variants (J1 and J4) were comparable to that obtained with the haemolytic strain (H), suggesting that in these cases HlyE export is somehow impaired (Table 4). To ensure that the isolated periplasmic fractions were free of cytoplasmic contamination the distribution of the cytoplasmic proteins aconitase and lipoamide dehydrogenase was determined by measuring enzyme activities. Significant quantities of both enzymes were only detected in the cytoplasmic fractions (60–170 units of aconitase activity min⁻¹ mg⁻¹ and 0.8–16.8 units of lipoamide dehydrogenase activity h⁻¹ mg⁻¹), but not in the periplasmic fractions (<2 units of aconitase min⁻¹ mg⁻¹ and <0.01–0.10 units of lipoamide dehydrogenase activity h⁻¹ mg⁻¹). Therefore, it was concluded that the periplasmic fractions were essentially free of cytoplasm, and that the non-haemolytic phenotype of strains J1 and J4 arises from an inability to export HlyE from the cytoplasm to the periplasm.

### The amino-terminal region of HlyE is required for export

A previous study identified two HlyE variants with single amino acid substitutions (N157H and Y165C) whose properties suggested that export of these proteins was impaired (Atkins et al., 2000). To investigate further which regions of HlyE are required for export from *E. coli* the *S. enterica* serovar Typhi hlyE was randomly mutagenized using the transposon TnphaA. Transposition of TnphaA allows random formation of in-frame fusions of the N-terminus of PhoA to a target protein, in this case HlyE. Target proteins that are secreted to the periplasm, or which are surface-exposed, or exported from the bacteria, are easily identified on PhoA indicator plates. Using TnphaA mutagenesis 4 out of 621 PhoA⁺ colonies lacked haemolytic activity. After DNA sequencing of one non-haemolytic isolate an in-frame insertion of PhoA after residue 179 of HlyE was detected. This insertion is at the end of the carboxyl-terminus of HlyE and removes the final 125 amino acids of HlyE. Interestingly, the previously described amino acids that apparently impair HlyE export were also located in zD (Atkins et al., 2000). Therefore, it is concluded that the carboxyl-terminus of HlyE is not required for HlyE export into the periplasmic space of *E. coli* and that zD plays an important role in this process.

### Transposon mutagenesis to identify genes potentially involved in HlyE export

Transposon mutagenesis was used to discover more about the mechanism by which HlyE is exported to the external milieu. It was not possible to use JM4660 as the host for mutagenesis because of its propensity to spawn non-haemolytic variants. Thus the non-haemolytic *E. coli* strain EC100 was co-transformed with a plasmid (pGS415; Table 1), expressing the *Actinobacillus pleuropneumoniae* FNR (HlyX) in order to confer a strong haemolytic phenotype, and the transposome complex EZ::Tn<KAN-2>Tnp. The transposon library was screened for colonies displaying impaired haemolysis on blood agar. Seven candidates emerged, from which genomic DNAs were prepared and the disrupted genes identified by DNA sequencing. Amongst these were two different examples of *hlyE* coding sequence lesions (Table 5). Five other insertion mutants that produced active HlyE protein, but had an impaired haemolytic phenotype, were unambiguously identified (Table 5). These included lesions in two genes of unknown function (*yyhl* and *yqeb*) and three genes with links to the cell envelope (*sfmA*, *waaG* and *tatC*). Because the *tatC*

### Table 4. Representative subcellular fractionation of haemolytic (H) and non-haemolytic (J1–J4) *E. coli* JM4660

Cultures of the indicated strains were grown under aerobic conditions for 16 h at 37°C in Lennox broth. The bacteria were collected, the cytoplasmic (C), periplasmic (P) and external medium (M) fractions were isolated, and haemolysins E (HlyE) activities were determined. HlyE activities are expressed as units min⁻¹ mg⁻¹ for the cytoplasmic and periplasmic fractions and as units min⁻¹ ml⁻¹ for the external medium (Rowe & Welch, 1994). Values quoted are the means and standard deviations of duplicate measurements from two cultures.

<table>
<thead>
<tr>
<th>Strain</th>
<th>HlyE activity</th>
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<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>H</td>
<td>5980 ± 380</td>
</tr>
<tr>
<td>J1</td>
<td>7270 ± 1040</td>
</tr>
<tr>
<td>J2</td>
<td>1950 ± 180</td>
</tr>
<tr>
<td>J3</td>
<td>690 ± 470</td>
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<tr>
<td>J4</td>
<td>5350 ± 290</td>
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gene encodes an essential component of the twin-arginine protein transloca
tion system, so called because conventional substrates contain an N-terminal (S/T)RRXFLK motif that is
required for recognition (Berks et al., 2000), and is involved in trafficking proteins to the periplasm, the effects of the
tatC lesion on HlyE export were further characterized. Estimation of the HlyE content of the tatC strain indicated that it possessed relatively more HlyE than the parent, suggesting an HlyE export defect (Table 5). To confirm that the impaired haemolytic phenotype was linked to transpo
don insertion into tatC, an independent tatC mutant was created and, as expected, after transformation with
pGS415 the resulting strain had an impaired haemolytic phenotype (not shown).

Inspection of the HlyE amino acid sequence did not reveal a potential Tat motif. However, not all Tat substrates possess a canonical motif and it was possible that HlyE might exit the cytoplasm via interaction with a bona fide Tat substrate. Therefore, the ability of the tatC mutant to export HlyE was investigated further. The approach taken was based on a recent study in which Tat-mediated export was investigated using GFP–SsrA fusions (DeLisa et al., 2002). Here an HlyE–SsrA fusion was used. The SsrA tag (AAN
DENYALAA) fused to the C-terminus of HlyE directs the cytoplasmic ClpXP and ClpAP proteases to rapidly and
efficiently degrade HlyE-SsrA. Thus, if a tatC mutant is impaired in the ability to export HlyE across the inner
membrane, HlyE–SsrA will be trapped in the cytoplasm, whereas the parental strain will be able to translocate HlyE–SsrA to the periplasm, where it will be degraded, whereas the parental strain will
be protected from Clp-mediated degradation. To prevent saturation of the proteases HlyE–SsrA was expressed with induction from a pBAD-based plasmid, pGS1699 (Table 1). As observed for native HlyE, colonies of the tat mutant expressing the HlyE-SsrA fusion were less haemo
ytic than the corresponding parental strain on blood agar. However, Western blotting of whole cells from liquid
cultures of the parent and the tat strain revealed that they possessed similar amounts of HlyE protein (not shown). The simplest explanation for these observations is that transfer of the HlyE–SsrA fusion from the cytoplasm to the periplasm is not impaired in the tat strain, but that export of HlyE–SsrA from the periplasm across the outer membrane must be more efficient in the parent to account for its enhanced haemolytic phenotype compared to the
 tatC mutant. A role for the outer membrane in HlyE export would be consistent with the reduced haemolysis associated with the disruption of genes linked to outer membrane structure–function (sfmA, tatC, and waaG) and the co-purification of HlyE with proteins of the inner and outer membrane (Table 5).

### DISCUSSION

The work described here was prompted by a report that the hlyE gene of an avian E. coli isolate, JM4660, encoded an Arg residue at a position occupied by Gly188 in the E. coli K-12 protein (Reingold et al., 1999), and the observation that growth of JM4660 on blood agar spawned non-haemolytic colonies. The experiments described lead to four conclusions. Firstly, that an Arg residue at position 188 abolishes HlyE activity. Secondly, that translocation of HlyE is not an intrinsic property of the protein but requires additional components. Thirdly, that translocation to the periplasm does not require the C-terminal region of HlyE. And fourthly, lesions in a subset of genes that affect outer membrane integrity impair HlyE export.

The reported substitution of Gly188 by Arg in the JM4660 HlyE protein was difficult to reconcile with the 3-D structure of HlyE and we have now shown that replacement of Gly188 by Arg inactivates E. coli K-12 HlyE, and that in fact contrary to a previous report (Reingold et al., 1999), Gly occupies this position in HlyE from JM4660. Nevertheless, the JM4660 hlyE gene encoded 76 amino acid

<table>
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<th>Gene disrupted</th>
<th>Blattner no.</th>
<th>Function</th>
<th>Relative haemolytic activity</th>
<th>Relative amount of HlyE</th>
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<tr>
<td>hlyE</td>
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<td>Haemolysin E</td>
<td>5</td>
<td>0</td>
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<tr>
<td>sfmA</td>
<td>B0530</td>
<td>Similarity to Salmonella Type IA pilin (precursor)</td>
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<td>86</td>
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<td>yqeB</td>
<td>B2875</td>
<td>Putative synthase</td>
<td>88</td>
<td>56</td>
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<tr>
<td>waaG</td>
<td>B3631</td>
<td>LPS core biosynthesis</td>
<td>111</td>
<td>100</td>
</tr>
<tr>
<td>tatC</td>
<td>B3839</td>
<td>Twin arginine translocase</td>
<td>295</td>
<td>1353</td>
</tr>
<tr>
<td>yjfiI</td>
<td>B4299</td>
<td>Putative transcription factor of the IclR family</td>
<td>57</td>
<td>63</td>
</tr>
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substitutions relative to the HlyE of \textit{E. coli} K-12, with the only extended region of conservation being the hydrophobic \( \beta \)-tongue that is required for interaction with target membranes (Wallace \textit{et al}., 2000). This, combined with the observation that the HlyE(Ala187\rightarrow Gly, Gly188\rightarrow Arg) variant is inactive, emphasizes the importance of this region for HlyE function. Indeed the overall properties of the K-12 and JM4660 proteins were remarkably similar, despite the differences in primary structure, which is particularly marked at the C-terminal end of the protein, where 17 of the last 35 residues are substituted. This latter observation is of interest because the JM4660 HlyE protein appeared to be more thermostable than the K-12 equivalent, and it has been shown that at least one substitution in the C-terminal region (Cys285\rightarrow Ser) dramatically reduces the thermostability of HlyE (Wallace \textit{et al}., 2000). Moreover, the HlyE protein from JM4660 was predominantly dimeric in solution and thus was much more homogeneous than the \textit{E. coli} K-12 equivalent, which has been observed in a variety of oligomeric states (monomer, dimer, and 8–12-mer) (Wallace \textit{et al}., 2000; Atkins \textit{et al}., 2000). In the crystal structure of water-soluble HlyE a dimer was formed by the head-to-tail arrangement of HlyE subunits such that the hydrophobic \( \beta \)-tongue and a second hydrophobic region consisting of residues Val89, Ala95, Ala96, Ile98, Leu99, Leu100, Ile115 and Ile282 located mainly within the C-terminus of \( \pi _{h} \) pack against each other to sequester these surfaces from the solvent (Wallace \textit{et al}., 2000). Comparing the JM4660 and K-12 proteins it is now clear that the residues of the \( \beta \)-tongue are highly conserved and its hydrophobic makeup is preserved. However, Ala95 and Leu99 of the second hydrophobic patch are replaced by Thr and Ser, respectively, in JM4660 HlyE. Inspection of the crystallographic dimer reveals that neither of these substitutions would generate any new interactions that could stabilize the HlyE dimer. There are relatively few specific interactions between the two molecules of the \textit{E. coli} K-12 HlyE crystallographic dimer, with only six direct protein–protein hydrogen bonds per subunit (Wallace \textit{et al}., 2000). However, examination of the differences in the amino acid sequence of the HlyE proteins did reveal three additional possible interactions in the avian \textit{E. coli} protein that could help stabilize the HlyE dimer (Fig. 5). Replacement of Asp114 by Thr may allow the formation of a hydrogen bond with the main chain N of Ala183; the double substitution Gln52\rightarrow Glu and Glu129\rightarrow Lys may promote the formation of a salt bridge; and replacement of Asp41 by Asn should remove a charge repulsion across the two-fold axis and lead to hydrogen bonding. Thus, it is suggested that these amino acid substitutions lead to additional interactions between subunits of the JM4660 HlyE and contribute to the formation of a more stable HlyE dimer. The observation that the JM4660 HlyE protein is predominantly dimeric in solution supports the suggestion that dimerization is used to maintain high solubility in aqueous media by hiding the hydrophobic lipid-binding amino acid residues from the solvent (Wallace \textit{et al}., 2000). Such a strategy is used by the water-soluble pore-forming toxin proaerolysin (Parker \textit{et al}., 1996). Comparison of the amino acid sequences of the \textit{E. coli} K-12 and JM4660 HlyE proteins reveals that most of the non-conservative amino acid substitutions are confined to the tail domain. This would be consistent with the head and central regions forming the core interactions between HlyE and target membranes, and between HlyE subunits in the assembled pore. Variation in the tail domain (predicted to extend from the membrane into the surrounding milieu) may contribute to antigenic variation in the HlyE proteins, which may be important if at least some HlyE is exported as a component of an outer-membrane vesicle (see below; Wai \textit{et al}., 2000, 2003a).

The observation that JM4660 spawned non-haemolytic variants that expressed active HlyE proteins, but failed to export that protein to the periplasm and ultimately to the

![Fig. 5. The HlyE dimeric interface. Stereodiagrams showing two \textit{E. coli} K-12 HlyE monomers, forming a dimer across the crystallographic two-fold axis, shown in (a) as main chain ribbons and in (b) in a space-filling representation. One monomer is shown in white, and the other in cyan; residues non-identical between \textit{E. coli} K-12 HlyE and avian \textit{E. coli} HlyE are highlighted on these monomers in green and dark blue, respectively. Sequence changes thought likely to alter the dimeric interaction are indicated in magenta. Both figures were created using MIDAS (Ferrin \textit{et al}., 1988).](http://mic.sgmjournals.org)
external medium, indicated that export is not an intrinsic property of the HlyE protein but requires additional components, which have been subverted in the non-haemolytic variants. The existence of an HlyE export system was suggested previously following the observation that the secretion of HlyE to the extracellular medium is independent of cytolytic activity (del Castillo et al., 2001). It has also been shown that, despite the lack of any previously recognized signal sequence, HlyE is transferred to the periplasm of E. coli, where it accumulates (Atkins et al., 2000; Ludwig et al., 1999; Wai et al., 2003b) and that this is independent of the type I, II, III, IV and V secretory systems (Wai et al., 2000). Thus, the process leading to the transfer of HlyE across the inner membrane is different from that used by other pore-forming toxins; e.g. proaerolysin translocation to the periplasm occurs co-translationally and requires the sec machinery (Parker et al., 1996). The finding that disruption of tatC, a component of the Tat pathway that mediates the translocation of folded proteins across the cytoplasmic membrane, impaired HlyE export suggested that Tat might direct HlyE to the periplasm. However, the absence of a Tat signal sequence within HlyE and the observation that HlyE apparently reaches the periplasm of a tatC mutant suggests an indirect role for Tat (see below). The failure of HlyE to reach the periplasm of the non-haemolytic JM4660 isolates characterized here implies that a distinct yet unknown mechanism to transfer HlyE to the periplasm exists. The TnphoA mutagenesis studies indicated that this export mechanism does not require the C-terminal region of HlyE, suggesting that the export sequence is located within the first 179 amino acids of HlyE. This is consistent with two HlyE variants with single amino acid substitutions (N157H and Y165C) that impair export (Atkins et al., 2000). Thus, it would appear that an intact $\alpha_2$ helix is important for HlyE translocation to the periplasm, consistent with the high level of amino acid conservation in this region of the protein (Fig. 2).

To attack a target cell HlyE has to be released from the periplasm into the extracellular milieu. It has been suggested that export of HlyE from the periplasm is mediated, at least in part, by outer-membrane blebbing (Wai et al., 2000, 2003a) and it is known that tat strains have defective outer membranes (Ize et al., 2003; Stanley et al., 2001). A membrane blebbing mechanism for HlyE export would be consistent with our observation that HlyE can be isolated in association with membrane proteins, and that mutations affecting the cell envelope (tatC, sfmA and waaG) can impair HlyE export. Thus, it would appear that the transposon insertions that impair HlyE export are linked to outer membrane structure–function. Hence, the integrity, composition and/or stability of the outer membrane are clearly important factors for the export of active HlyE.

In conclusion, the results reported here demonstrate the presence of a functional HlyE toxin in an avian E. coli isolate that lacks other haemolysins. Characterization of the isolated protein emphasized the importance of the $\beta$-tongue in HlyE function and provided evidence for dimerization as a strategy for improving toxin solubility. Moreover, the characterization of non-haemolytic variants of E. coli JM4660 and transposon mutagenesis indicates that export of active HlyE to the periplasm does not require the C-terminal region of HlyE and is not an intrinsic property of the protein itself but requires additional components, including the integrity of the outer membrane.

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