The production of HlyA toxin by *Proteus penneri* strains

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Summary. Twelve diverse strains of *Proteus penneri* of clinical origin all produced a calcium-dependent haemolysin, unlike most other *Proteus* spp. In most strains the haemolysin was secreted into the medium during early exponential growth and lysed not only of a variety of erythrocyte types from several animals including man, but also human neutrophils and human embryo lung fibroblasts. The haemolysin was a protein of 107 kDa, the same size as *Escherichia coli* HlyA, and it reacted with antiserum to *E. coli* HlyA. Because of its similarity in size, antigenicity and range of action to the HlyA virulence factor of *E. coli*, *P. penneri* HlyA is believed to be an important virulence factor for this organism. It was degradable by an EDTA-sensitive protease—probably the IgA protease—to inactive fragments. The interaction of *P. penneri* HlyA and IgA protease *in vivo* and the origin of HlyA, which has now been found in many diverse bacteria, are discussed.

Introduction

RTX toxins are a family of calcium-dependent, pore-forming, cytolytic toxins produced by diverse bacteria that act on different cells in different hosts. Thus *Escherichia coli* α -haemolysin, *Actinobacillus pleuropneumoniae* haemolysin and *Bordetella pertussis* adenylate cyclase-haemolysin lyse both erythrocytes and a wide range of nucleated cells from many different animals including man.^{1–3} On the other hand, the leucotoxin of *Pasteurella haemolytica* and *A. actinomycetemcomitans* acts only on neutrophils, monocytes and macrophages of ruminant animals and selected primates including man, and is without action on erythrocytes.^{4,5} These toxins are so named (Repeats in ToXin) because, despite the diversity in the susceptibility of host cells to them, they have a common structural feature of repeats (usually in tandem) of a nine amino acid, glycine-rich sequence (Leu/Ile/Phe-X-Gly-Gly-X-Gly-Asn/Asp-Asp-X) that is thought to be involved in the binding of calcium and interaction with target receptors.⁶

RTX toxins are important virulence factors, not only because they destroy important defence cells of the body, but also because, at concentrations too low to cause cell lysis such as would occur in the body at sites distant from the infecting organism, they impair various functions of host cells involved in the immune response.⁷

Proteus penneri, the bacterium formerly known as “indole-negative *P. vulgaris*” or “*P. vulgaris* biogroup 1”,⁸ although less commonly isolated from clinical specimens than *P. mirabilis* and *P. vulgaris*, has, nevertheless, been associated with infections of the

urinary tract⁹ and wounds¹⁰ and has been isolated from the faeces of both healthy and sick individuals.¹¹ Potential virulence factors already known to be produced by *P. penneri* include urease,¹² IgA protease^{13,14} and type 3 fimbriae associated with MR/K haemagglutinins, of at least two antigenic types.¹⁵ The work presented here examined the production of a haemolysin antigenically related to the *E. coli* RTX toxin α -haemolysin (HlyA) that could be an additional factor to be considered in the virulence of this organism.

Materials and methods

Bacteria

The 12 strains of *P. penneri* studied were clinical isolates from different patients in three countries (table). Those prefixed E were from patients with diarrhoea and those prefixed H from healthy volunteers and were donated by Dr H. E. Müller. Strain T77 was isolated by Dr O. Ang in Istanbul. The Dundee strains were from elderly women with haematuria. *E. coli* strain 519, serotype O4, isolated from urine, was a known producer of the calcium-dependent α -haemolysin, HlyA.^{16,17} *P. mirabilis* U6450 was a strain from urine that produced the calcium-independent cell-bound haemolysin Hpma.¹⁷ *P. vulgaris* strain 76362/79 was isolated from an infected burn graft. It produced both Hpma and HlyA.^{17–19} The strains were characterised by standard biochemical tests²⁰ and maintained on Nutrient Agar (Oxoid CM3) slopes in screw-capped bottles at 4°C.

Haemolysin production

An inoculum (100 μ l) of a Nutrient Broth (Oxoid

Table. Characteristics of the *P. penneri* strains studied

Strain no.	Proticene production (p) /sensitivity (s) type	Source	Origin
E 46	p0/s2, 3, 5	Faeces	Germany
E 100	p0/s2, 3, 5	"	"
E 180	p0/s4	"	"
E 587	p0/s2, 3, 5	"	"
E 1125	p0/s12	"	"
E 1152	p0/s12	"	"
E 1263	p0/s12	"	"
H 1209	p0/s2, 3, 5	"	"
H 1360	p0/s12	"	"
T 77	p0/s9, 12	Thorax wound	Istanbul, Turkey
05665V	p0/s4, 12	Urine	Dundee, UK
16803Z	p0/s0	"	"

CM67) culture of each strain, which had been incubated overnight at 37°C, was added to 10 ml of nutrient broth and incubated with shaking at 37°C. At intervals, samples of culture were removed for determination of growth (absorbance at 550 nm). In addition 50 µl of the culture, or the supernate after centrifugation of the culture sample for 2 min at 11 600 *g*, was added to a microcentrifuge tube holding 1 ml of a washed suspension of erythrocytes (usually horse erythrocytes), 2% v/v in saline with and without 20 mM CaCl₂. After inverting the tubes to mix the contents and incubating them in a water bath at 37°C for 15 min, the tubes were centrifuged at 11 600 *g* for 30 s to pellet the erythrocytes and bacteria. The amount of free haemoglobin in the supernate was measured by its absorbance at 540 nm against a blank of water and this indicated the haemolytic activity of the sample.

Effect of culture supernates on human embryo lung (HEL) fibroblasts

At intervals, 0.5 ml of the culture of each strain, growing under the conditions as for haemolysin production, was centrifuged at 11 600 *g* for 2 min. A 50-µl portion of the clear supernate was removed and assayed for haemolytic activity as above and 50 µl of the supernate was also added to the wells of microtitration plates bearing a confluent growth of HEL fibroblasts in Eagle's maintenance medium. The plates were incubated at 37°C and examined under a plate microscope for cytopathic effects after 24 and 48 h.

Effect of haemolysin on neutrophils

Haemolysin-induced damage to neutrophils was examined by assaying for lactate dehydrogenase released from damaged neutrophils. Lactate dehydrogenase causes the following reaction to take place: pyruvate + βNADH⁺ + H⁺ → lactate + βNAD⁺. The formation of βNAD⁺ can be followed by measuring the decrease in absorption at 340 nm.

Neutrophils were isolated from the heparinised blood of healthy volunteers by centrifugation at 400 *g*

for 30 min through an equal volume of Lymphoprep (Nycomed, Oslo; specific gravity 1.077) lying above an equal volume of Ficoll-hypaque (specific gravity 1.119). The neutrophils which collected at the Lymphoprep-Ficoll-hypaque interface were collected, washed and resuspended to 10⁶ cells/ml in Tris-buffered saline (TBS; NaCl 0.85% in 50 mM Tris-HCl; pH 7.5). The average purity of the preparations was > 95% neutrophils.

For the test, 200-µl amounts of active haemolysin from the supernate of a nutrient-broth culture of *P. penneri* strain E 1125 incubated for 3 h was added to 200-µl amounts of neutrophils in TBS in the presence (test reaction) and absence (control reaction) of 20 mM CaCl₂. The mixtures were incubated in a water bath at 37°C. At timed intervals, samples of equal volume of a control and test reaction were removed and added simultaneously to separate cuvettes each holding 600 µl of TBS to which had been added 30 µl of a fresh solution of reduced β-nicotinamide adenine dinucleotide (β-NADH; 3.5 mg/ml in TBS) and 30 µl of fresh sodium pyruvate solution (11 mg/ml in TBS) and the 340 nm absorption of the reaction in each cuvette was recorded with time. A further enzyme control reaction consisted of 200 µl of neutrophils in TBS treated with 200 µl of Triton X100 (2% in TBS) for 5 min at room temperature to cause total lysis of neutrophils. The amount of lactate dehydrogenase from this reaction was measured as above.

SDS-PAGE of P. penneri haemolysins

Strains were cultured as for haemolysin production and, when found to be actively producing haemolysin, 1-ml samples were removed and centrifuged at 11 600 *g* for 2 min. The clear supernate was transferred to a microcentrifuge tube and lysozyme, as a carrier protein, was added to it to a final concentration of 100 µg/ml. Trichloroacetic acid (TCA) was added to the mixture to 10% w/v to precipitate proteins. After 10 min, the mixture was centrifuged at 11 600 *g* for 2 min, the supernate was discarded and the tube

contents were drained well. Twenty-five μ l of 1 M Tris was added to the precipitate to neutralise contaminating residual acid. The precipitate was resuspended and dissolved after the further addition of 25 μ l of sample buffer (0.125 M Tris-HCl, pH 6.8, SDS 4%, glycerol 20%, mercaptoethanol 10% and a trace of bromophenol blue dye). The samples were stored at -20°C . Before electrophoresis, samples were boiled for 5 min and 25 μ l was loaded on to a stacking gel of acrylamide 4% in 0.125 M Tris-HCl, pH 6.8, SDS 0.1% over a resolving gradient gel of acrylamide 5–15% in 0.375 M Tris-HCl, pH 8.8, SDS 0.2%. The upper tank buffer was 0.053 M Tris, 0.052 M glycine, SDS 0.1%, pH 8.9, and the lower tank buffer was 0.1 M Tris-HCl, SDS 0.1%, pH 8.1. Electrophoresis was performed in the cold at 25 mA until the tracking dye front reached the bottom of the gel. Gels were then either stained overnight with Coomassie Brilliant Blue R250 0.125% in water containing methanol 50% and acetic acid 10% and destained in several changes of water containing methanol 10% and acetic acid 10%, or prepared for immunoblotting.

Immunoblotting of haemolysins

After PAGE of haemolysin-containing culture supernates and reference protein mol. wt markers, the separated proteins were transferred to nitrocellulose membranes by electrophoresis in methanol 10% containing 25 mM Tris and 190 mM glycine at 4°C for 16 h at 10 V. The membrane was stained in Ponceau S dye 0.2% in TCA 3% and the positions of the reference proteins were marked. After thoroughly washing the membrane in Tris-saline (TS; 10 mM Tris-HCl, pH 7.4, 150 mM NaCl), the membrane was agitated for 2 h in TS containing dried milk powder (Marvel) 5% and then washed in several changes of wash buffer (TS containing Tween 20 0.05%). The membrane was then agitated for 2 h at room temperature in rabbit antibody to purified *E. coli* α -haemolysin HlyA (kindly donated by Dr C. Hughes, Department of Pathology, University of Cambridge) diluted 1 in 5000 in wash buffer. After washing the membrane in several changes of wash buffer, it was agitated for 2 h at room temperature in alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1 in 400 in wash buffer and subsequently washed in several changes of wash buffer. The immunoblot was developed in 30 ml of buffer (100 mM NaCl, 5 mM MgCl_2 , 100 mM Tris-HCl, pH 9.5) containing 100 μ l of bromochloro-indolyl phosphate (50 mg/ml in dimethyl formamide) and 200 μ l nitro-blue tetrazolium (50 mg/ml in dimethyl formamide 70% in water).

Results

All 12 strains studied had the characteristic biochemical properties of *P. penneri* and all produced tryptophan deaminase and urease, and fermented both

glucose and maltose; however, none was able to produce ornithine decarboxylase or indole nor to ferment lactose, mannose, mannitol, adonitol, inositol or salicin, nor to degrade aesculin.

Kinetics of haemolysin production

During exponential growth in nutrient broth at 37°C , each strain produced sufficient activity to lyse horse erythrocytes. The haemolysin was dependent on calcium for activity because there was very little erythrocyte lysis when calcium was absent or the calcium chelator EGTA (5 mM in Tris buffer, pH 7) was present. For each strain, as the phase of growth moved from the exponential to the stationary, the haemolytic activity declined until none was detectable. For strain E 46 (fig. 1), and strains E 100, E 587 and H 1209 that behaved similarly, the calcium-dependent haemolysin was cell-associated, for no haemolytic activity was detected in culture supernates in the presence of calcium. However, for strain E 1125 (fig. 2) and seven others, E 180, E 1152, E 1263, H 1360, T 77, 05665V and 16803Z which behaved similarly, the calcium-dependent haemolysin was secreted into the culture medium.

Activity of haemolysin on different erythrocyte types

The activity of the supernates from 3-h cultures of the above eight strains that produced cell-free haemo-

Fig. 1. The kinetics of growth (▲—▲; 550 nm absorbance) of *P. penneri* strain E 46 in nutrient broth at 37°C and the haemolytic activity of the culture in the presence of 20 mM CaCl_2 (●—●) and in its absence (▼—▼) and of the culture supernate (■—■) in the presence of calcium.

Fig. 2. The kinetics of growth (Δ — Δ ; 550 nm absorbance) of *P. penneri* strain E 1125 in nutrient broth at 37°C and the haemolytic activity of the culture ($\bullet$ — $\bullet$) and the culture supernate ($\blacksquare$ — $\blacksquare$) in the presence of 20 mM CaCl_2 and of the culture (∇ — ∇) and the culture supernate ($\blacklozenge$ — $\blacklozenge$) in the absence of CaCl_2 .

lysin was tested against a range of erythrocytes from different animals. In the presence of calcium, but not in its absence, all lysed pig, ox, rat, guinea-pig, fowl and human O erythrocytes as well as horse erythrocytes.

Activity of haemolysin on human embryo lung (HEL) fibroblasts

Samples (50 μl) of culture supernates of all the strains taken at different stages of the growth cycle were simultaneously assayed for haemolytic activity to horse erythrocytes in the presence of calcium, and also added to HEL fibroblasts. After incubation for 24 h, a cytopathic effect (CPE) on the HEL monolayer was given only by those culture supernates with haemolytic activity. The extent of the CPE appeared to correlate directly with the haemolytic activity of the supernate. None of either the supernates of the strains forming only cell-associated haemolysin, or the supernates of the other haemolytic strains sampled either before haemolysin production was detectable or after haemolysin activity was lost in the stationary phase of growth, caused a CPE, even when incubation was extended to 48 h.

Activity of haemolysin on neutrophils

Addition of culture supernates containing haemolysin to neutrophils in the absence of calcium appeared to have little or no deleterious effect for, even after incubation for 1 h, there was evidence of release of

Fig. 3. The effect of haemolysin from *P. penneri* strain E 1125 in the presence (—) and absence (---) of 20 mM CaCl_2 ; and of Triton X100 (-----), on the release of lactate dehydrogenase (measured as a decline in absorption at 340 nm) from human neutrophils after incubation with haemolysin for 15 min (∇), 30 min ($\blacksquare$) and 1 h ($\bullet$) at 37°C.

Fig. 4. PAGE of concentrated culture supernates of: lanes 2–5, non-secreting strains of *P. penneri* H 1209, E 587, E 100 and E 46 respectively, which lacked calcium-dependent haemolytic activity; lanes 6–11, haemolysin-secreting strains of *P. penneri* E 1263, E 180, E 1125, T 77, 16803Z and E 1152 respectively, which had calcium-dependent haemolytic activity; lanes 12 and 13, strains E 1263 and E 180 respectively, before secretion of haemolysin into the supernate. Lanes 1 and 14 contained low- and high-mol. wt protein markers respectively. The position of the calcium-dependent haemolysin is marked (→).

only a very small amount of lactate dehydrogenase from the neutrophils (fig. 3). This was not greater than that given when neutrophils were incubated with sterile nutrient broth containing 20 mM CaCl_2 and is thought to be the outcome of non-specific deterioration of a small proportion of the neutrophils during the course of the experiment.

In the presence of haemolysin and calcium, however, there was release of lactate dehydrogenase, indicating neutrophil damage. After incubation of neutrophils with haemolysin for 1 h, virtually all the neutrophils were damaged because the amount of lactate dehydrogenase released was almost the same as that given by the neutrophils lysed by Triton X100 (fig. 3). These results indicated that the calcium-dependent haemolysin damaged neutrophils.

SDS-PAGE of haemolysin

In an attempt to determine the size and nature of the calcium-dependent haemolysin of *P. penneri*, active haemolytic supernates from 3-h cultures of six of the strains secreting haemolysin were concentrated and analysed by SDS-PAGE. As controls, culture supernates taken before haemolysin was secreted from the cell, together with culture supernates from 3-h cultures of the four non-secreting haemolytic strains, were also concentrated and analysed on the same gel. The stained gel (fig. 4) showed the presence of a number of

proteins common to the supernates of both the secreting and non-secreting haemolytic strains. However, one protein of *c.* 107 kDa was present in all the preparations where there had been active haemolysin, yet was absent from all the preparations sampled before haemolysin was detectable in the culture supernate and from those of the non-secreting haemolytic strains. This suggested that the secreted calcium-dependent haemolysin was a protein of *c.* 107 kDa.

Immunoblot analysis of haemolysins

The supernates from 3-h nutrient-broth cultures of all the *P. penneri* strains, the known calcium-dependent haemolysin (HlyA) toxin producers *E. coli* strain 519 and *P. vulgaris* 76362/79, and *P. mirabilis* U6450 which produces the calcium-independent cell-bound haemolysin HpmA, together with those from 6.5-h and 16-h cultures of *P. penneri* strains E 180 and E 1125, were assayed for calcium-dependent haemolytic activity and were also concentrated and electrophoresed on polyacrylamide gels. After Western blotting, the blots were allowed to react with rabbit antibody to *E. coli* HlyA and subsequently with alkaline phosphatase-conjugated antibody to rabbit IgG and then developed.

Every *P. penneri* strain capable of forming calcium-dependent haemolysin in culture supernates was found to produce a protein in the culture supernate which,

Fig. 5. Immunoblot of concentrated supernates with active calcium-dependent haemolytic activity from 3-h nutrient broth cultures of: lane 1, *E. coli* 519; 2, *P. vulgaris* 76362/79; 3, *P. penneri* E 180; 6, *P. penneri* E 1125; 9, *P. penneri* E 1263; 10, *P. penneri* H 1360; 15, *P. penneri* E 1152; 16, *P. penneri* T 77; 17, *P. penneri* 16803Z; 18, *P. penneri* 05665V. Concentrated supernates whose haemolytic activity was lost through culture for 6.5 h and 16 h from *P. penneri* E 180 (lanes 4 and 5) and E 1125 (lanes 7 and 8). Concentrated culture supernates which lacked calcium-dependent haemolytic activity from 3-h cultures of (lanes 11–14) non-secreting *P. penneri* strains H 1209, E 587, E 100 and E 46 respectively and (lane 19) *P. mirabilis* U6450. Positions of 116-, 97.4-, 66- and 42.7-kDa mol.-wt markers are shown on the left.

Fig. 6. Immunoblot of supernates of a nutrient-broth culture of *P. penneri* E 1125 taken after incubation at 37°C for 3, 4, 5, 6, 8 and 16 h, respectively (lanes 1–6), and concentrated, and supernates taken after incubation at 37°C for 3, 4, 5 and 6 h, then supplemented with EDTA to 10 mM and further incubated for 16 h at 37°C before concentration (lanes 7–10). After electrophoresis, the preparations were incubated with rabbit IgG to *E. coli* HlyA haemolysin and subsequently with alkaline phosphatase-conjugated anti-rabbit IgG before development. The positions of protein mol.-wt markers are shown on the left.

when it had haemolytic activity, was of *c.* 107 kDa. This protein reacted with antibody to *E. coli* HlyA haemolysin (fig. 5). The protein was of the same size as the haemolysin produced by the *E. coli* and *P. vulgaris* strains which also reacted with the antibody.

Analysis of the supernates of cultures of *P. penneri* strains E 180 and E 1125 incubated until the calcium-

dependent haemolytic activity of the supernate was just lost, showed the absence of the 107-kDa protein but the presence of a 44-kDa protein which reacted with the antibody to *E. coli* HlyA haemolysin (fig. 5, lanes 4 and 7). This is believed to be a protease-cleaved fragment of the haemolysin that had no haemolytic activity. After incubation of cultures for 16 h this

fragment was not found (fig. 5, lanes 5 and 8). It is believed to have been further degraded to small peptides.

No protein capable of reacting with *E. coli* HlyA antiserum was found in the supernates of the *P. penneri* strains that did not form calcium-dependent, cell-free haemolysin or in that of *P. mirabilis* U6450 (fig. 5, lanes 11–14 and 19, respectively).

These results confirmed that the 107-kDa protein found in the supernates of haemolysin-secreting strains of *P. penneri* was the calcium-dependent haemolysin and that it was both the same size as *E. coli* α -haemolysin (HlyA) and antigenically related to it. Furthermore, the decline and eventual loss of haemolytic activity found when cultures entered the late logarithmic phase of growth was the outcome of proteolytic degradation of the haemolysin to an intermediate protein of 44 kDa and subsequently to much smaller fragments.

Further study of the products formed when the haemolysin of *P. penneri* strain E 1125, which reacted with antibody to *E. coli* HlyA, began to decline in activity revealed (fig. 6) that many degraded intermediates were formed. However, degradation of the haemolysin was prevented to a substantial degree if 10 mM EDTA was present. This suggested that the degradation was the result of a metallo-proteinase.

Discussion

Among the *Proteaeae*, two different chromosomally determined haemolysins, HpmA and HlyA, have been found to be produced. HpmA, a protein of 166 kDa,¹⁹ is a calcium-independent haemolysin produced by virtually all strains of *P. mirabilis* and most strains of *P. vulgaris*.^{18,19} It is cytolytic not only for erythrocytes but also for a wide range of eukaryotic cell types including human renal proximal tubular epithelial cells.^{21,22}

On the other hand, HlyA, a protein of 107 kDa, is a calcium-dependent haemolysin and, though similarly active against a wide range of erythrocyte types and eukaryotic cells,²³ (and unpublished observations) is extremely toxic for mice²⁴ and reacts with antiserum to *E. coli* HlyA.²³ It is produced by 30–56% of *Morganella morganii* strains.^{18,25} However, HlyA production by *P. mirabilis* strains has never been reported and is also extremely rare in *P. vulgaris*, although a few strains that produce both HpmA and HlyA have been isolated.^{18,19} The genes determining HlyA production in both *P. vulgaris* and *M. morganii* are structurally and functionally very similar to those determining HlyA production in *E. coli*.¹⁷

The results presented here suggest that, unlike the

situation in *P. mirabilis* and *P. vulgaris*, HlyA production is common in *P. penneri*, as it was found in all the randomly selected diverse strains examined. In *E. coli*, HlyA secretion is a complex process involving a secretion signal at the C-terminus of HlyA and the products of *hlyB*, *hlyD* and *tolC* genes.^{26,27} The four *P. penneri* strains that did not secrete haemolysin were all of the same proticene production-sensitivity type that was unique to them. It is probable that they have a common defect in one or more of these determinants.

Functionally, the secreted haemolysin of *P. penneri* strains had similar properties to that of *E. coli* HlyA. It was without activity in the absence of calcium or the presence of the calcium chelator EDTA, indicating that it required calcium for activity. In the presence of calcium it caused lysis or damage to both erythrocytes from many different animals and also eukaryotic cells, including neutrophils and human lung fibroblasts. Moreover, it was indistinguishable in size and antigenicity from the HlyA of *E. coli* and *P. vulgaris*.

The production of HlyA by *P. penneri* was also similar to that in *E. coli*. It occurred early in logarithmic growth and haemolytic activity declined rapidly as the cells entered the stationary phase of growth. In *E. coli* the reason for the lability of HlyA is not fully known, although it is likely to be because of aggregation²⁸ and possibly also proteolytic breakdown. Although, in this study, the formation of inactive aggregates of *P. penneri* haemolysin was not sought, there was clear evidence for the proteolytic degradation of the haemolysin to inactive fragments which was coincident in time with the decline in haemolytic activity of the cultures. The inhibition of this degradation by EDTA suggests that the enzyme involved is the EDTA-sensitive IgA protease of *P. penneri*.^{13,14}

If it is the IgA protease of *P. penneri* that degrades *P. penneri* HlyA *in vitro*, this interference of one virulence factor with another may not occur *in vivo*. The production and secretion of haemolysin before that of the protease, together with its rapid binding to and activity on target cells that are unlikely to be the substrate of the protease, may permit both virulence factors of *P. penneri* to act together, rather than in opposition, *in vivo* to cause disease.

Ever since it was found that the GC composition of the *E. coli* haemolysin operon was 40 mol%,²⁹ some 10% lower than that of the total genome, consideration has been given as to the origin of HlyA. The genus *Proteus* was thought to be a likely contender until it was realised that HlyA production is rarely ever seen among the most frequently isolated species of *Proteus*.¹⁹ However, the finding here, that HlyA production is common in *P. penneri* whose genomic DNA content is 38 mol% GC,⁸ suggests that this organism is a strong contender as the source from which HlyA originated.

References

1. Bhakdi S, Greulich S, Muhly M *et al.* Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. *J Exp Med* 1989; **169**: 737–754.
2. Kamp EM, van Leengoed LAMG. Serotype-related differences in production and type of heat-labile hemolysin and heat-

- labile cytotoxin of *Actinobacillus (Haemophilus) pleuropneumoniae*. *J Clin Microbiol* 1989; **27**: 1187–1191.
3. Glaser P, Sakamoto H, Bellalou J, Ullmann A, Danchin A. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. *EMBO J* 1988; **7**: 3997–4004.
 4. Shewen PE, Wilkie BN. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infect Immun* 1982; **35**: 91–94.
 5. Tsai C-C, Shenker BJ, Dirienzo JM, Malamud D, Taichman NS. Extraction and isolation of leukotoxin from *Actinobacillus actinomycetemcomitans* with polymyxin B. *Infect Immun* 1984; **43**: 700–705.
 6. Welch RA. Pore-forming cytotoxins of Gram-negative bacteria. *Mol Microbiol* 1991; **5**: 521–528.
 7. Cavalieri SJ, Snyder IS. Effect of *Escherichia coli* alpha-hemolysin on human peripheral leukocyte function *in vitro*. *Infect Immun* 1982; **37**: 966–974.
 8. Hickman FW, Steigerwalt AG, Farmer JJ, Brenner DJ. Identification of *Proteus penneri* sp. nov., formerly known as *Proteus vulgaris* indole negative or as *Proteus vulgaris* biogroup 1. *J Clin Microbiol* 1982; **15**: 1097–1102.
 9. Krajden S, Fuksa M, Lizewski W, Barton L, Lee A. *Proteus penneri* and urinary calculi formation. *J Clin Microbiol* 1984; **19**: 541–542.
 10. Krajden S, Fuksa M, Petrea C, Crisp LJ, Penner JL. Expanded clinical spectrum of infections caused by *Proteus penneri*. *J Clin Microbiol* 1987; **25**: 578–579.
 11. Müller HE. Occurrence and pathogenic role of *Morganella-Proteus-Providencia* group bacteria in human feces. *J Clin Microbiol* 1986; **23**: 404–405.
 12. Mobley HLT, Jones BD, Penner JL. Urease activity of *Proteus penneri*. *J Clin Microbiol* 1987; **25**: 2302–2305.
 13. Senior BW, Albrechtsen M, Kerr MA. A survey of IgA protease production among clinical isolates of *Proteaceae*. *J Med Microbiol* 1988; **25**: 27–31.
 14. Loomes LM, Senior BW, Kerr MA. Proteinases of *Proteus* spp.: purification, properties, and detection in urine of infected patients. *Infect Immun* 1992; **60**: 2267–2273.
 15. Yakubu DE, Old DC, Senior BW. The haemagglutinins and fimbriae of *Proteus penneri*. *J Med Microbiol* 1989; **30**: 279–284.
 16. Knapp S, Hacker J, Then I, Müller D, Goebel W. Multiple copies of hemolysin genes and associated sequences in the chromosome of uropathogenic *Escherichia coli* strains. *J Bacteriol* 1984; **159**: 1027–1033.
 17. Koronakis V, Cross M, Senior B, Koronakis E, Hughes C. The secreted hemolysins of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii* are genetically related to each other and to the alpha-hemolysin of *Escherichia coli*. *J Bacteriol* 1987; **169**: 1509–1515.
 18. Senior BW, Hughes C. Production and properties of haemolysins from clinical isolates of the *Proteaceae*. *J Med Microbiol* 1988; **25**: 17–25.
 19. Swihart KG, Welch RA. The HpmA hemolysin is more common than HlyA among *Proteus* isolates. *Infect Immun* 1990; **58**: 1853–1860.
 20. Senior BW, Leslie DL. Rare occurrence of *Proteus vulgaris* in faeces: a reason for its rare association with urinary tract infections. *J Med Microbiol* 1986; **21**: 139–144.
 21. Swihart KG, Welch RA. Cytotoxic activity of the *Proteus* hemolysin HpmA. *Infect Immun* 1990; **58**: 1861–1869.
 22. Mobley HLT, Chippendale GR, Swihart KG, Welch RA. Cytotoxicity of the HpmA hemolysin and urease of *Proteus mirabilis* and *Proteus vulgaris* against cultured renal proximal tubular epithelial cells. *Infect Immun* 1991; **59**: 2036–2042.
 23. Eberspächer B, Hugo F, Pohl M, Bhakdi S. Functional similarity between the haemolysins of *Escherichia coli* and *Morganella morganii*. *J Med Microbiol* 1990; **33**: 165–170.
 24. Emödy L, Vörös S, Pal T. Alpha-haemolysin, a possible virulence factor in *Proteus morganii*. *FEMS Microbiol Lett* 1982; **13**: 329–331.
 25. Vörös S, Senior BW. New O antigens of *Morganella morganii* and the relationships between haemolysin production, O antigens and morganocin types of strains. *Acta Microbiol Hung* 1990; **37**: 341–349.
 26. Wandersman C, Delepelaire P, Tol C, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc Natl Acad Sci USA* 1990; **87**: 4776–4780.
 27. Koronakis V, Stanley P, Koronakis E, Hughes C. The HlyB/HlyD-dependent secretion of toxins by Gram-negative bacteria. *FEMS Microbiol Immunol* 1992; **105**: 45–54.
 28. Ostolaza H, Bartolomé B, Serra JL, de la Cruz F, Goñi FM. α -Hemolysin from *E. coli*: purification and self-aggregation properties. *FEBS Lett* 1991; **280**: 195–198.
 29. Felmler T, Pellett S, Welch RA. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J Bacteriol* 1985; **163**: 94–105.